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# DETERMINANTS OF MOTOR UNIT SIZE IN NORMAL AND REINNERVATED TIBIALIS ANTERIOR MUSCLES OF THE RAT.

BY

JOANNE ELIZABETH TÖTÖSY de ZEPETNEK
(née TOMS)

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN
PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
THE DEPARTMENT OF PHARMACOLOGY
(NEUROSCIENCE)

EDMONTON, ALBERTA
SPRING, 1990

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April 23, 1990

*Thus, whatever rose towards the light is left to sink unnamed: a shape that passes slowly through a dream. Waking, all we remember is the awesome presence, while a shadow lying dormant in the twilight whispers from the other side of reason; I am here. I wait.*

*Timothy Findley*

*Famous Last Words*

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled,

DETERMINANTS OF MOTOR UNIT SIZE IN NORMAL AND REINNERVATED TIBIALIS ANTERIOR MUSCLES OF THE RAT.

submitted by JOANNE ELIZABETH TOTOSY de ZEPETNEK (née TOMS)

in partial fulfilment of the requirements for the degree

of DOCTOR OF PHILOSOPHY

in NEUROSCIENCE

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Thomas P Martin ..... Dr. Thomas P. Martin

Date: April 13, 1990

## ABSTRACT

Motor unit (MU) force production depends on the number of muscle fibres innervated per motoneurone (innervation ratio, IR), mean muscle unit fibre cross-sectional area (A) and force generating capacity per unit area (specific tension, ST). It remains controversial to what extent differences in the number of branches per motoneurone (IR), compared to muscle properties (A and ST) account for the observed range of unit force. This study examined the relative contributions of these neural and muscle factors to MU force in normal tibialis anterior (TA) muscles and the plasticity of the neuromuscular system after reinnervation. MUs were isolated, characterized physiologically and histochemically for direct and indirect estimations of IR, A and ST, and morphometric analysis of muscle units.

We found that IR varies between 57 and 202 and accounts for 39% of the range in force. There is also a systematic variation of force with A. In contrast, ST averages 2.35 ± 0.04 kg/cm$^2$ for all units, irrespective of physiological type. The range of unit force in reinnervated muscles was similar to normal. IR varied between 63 and 374 and accounted for 69% of the force range, however A showed little variation with force indicating that neural plasticity plays a significant

role in re-establishing the wide range of force after reinnervation. The more limited muscle plasticity was also evident from the poor correlation of force and fatigability in reinnervated units.

The spatial territory of muscle units, defined as the cross-sectional area bounded by the outermost muscle fibres, varied systematically with IR and unit force. In reinnervated units, these territories were reduced by a factor of 8- to 10-fold as a result of clumping of muscle fibres, but the relationship between unit territory, IR and force remained.

It is widely recognized that unit force is correlated with motoneurone size in normal and reinnervated muscles. This study shows that motoneurone branching is a significant factor which determines the range of MU force and the correlation with motoneurone size. The results suggest further that neuronal branching is size dependent, as originally suggested by Ramon y Cajál in 1909.

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## LIST OF ABBREVIATIONS

A = mean muscle unit fibre cross-sectional area

AL = adductor longus

ATPase = adenosine triphophosphatase stain

CFI = cumulative fatigue index

CSA = cross-sectional area

2-DG = 2-deoxyglucose

EDL = extensor digitorum longus muscle

EMG = electromyogram, or electromyograph

1/2FT = half fall time

FCR = flexor carpi radialis muscle

FDL = flexor digitorum longus muscle

FF = fast fatigable motor unit

FG = fast glycolytic muscle fibre

FI = fast fatigue intermediate motor unit

Fint = fast intermediate muscle fibre

FOG = fast oxidative, glycolytic muscle fibre

FR = fast fatigue resistant motor unit

HRP = horseradish peroxidase

Hz = hertz

IEMG = integrated EMG

ip = intraperitoneal

ipi = interpulse interval

IR = innervation ratio

kg = kilograms

LG = lateral gastrocnemius muscle

$\mu m^2$ = square micrometers

$\mu V$ = microvolts

m = slope

mm = millimeters

mN = millinewtons

MG = medial gastrocnemius muscle

ms = milliseconds

MU = motor unit

mV = millivolts

NADH = nicotinamide adenine dinucleotide diaphorase stain

N-CAM = neural cell adhesion molecule

PAS = periodic acid-Schiff stain

PFI = progressive fatigue index

PG = posterior gracilis muscle

PL = peroneus longus muscle

r = correlation coefficient from linear regression analysis

RIR = relative innervation ratio

1/2RT = half rise time

S = slow motor unit

SD = standard deviation

SE = standard error

SO = slow oxidative muscle fibre

ST = specific tension

TA = tibialis anterior muscle

TP = tibialis posterior muscle

TTP = time to peak twitch force

# CHAPTER 1

# GENERAL INTRODUCTION

## 1.I Introduction to the Thesis Topic

Differences in cell body size in the central nervous system were pointed out nearly a century ago by Ramon y Cajal (1909) after an extensive histological examination of nervous tissue. His observations led him to conclude "...that the size of a cell is related to the diameter of its axis cylinder and especially to the number and thickness of its collaterals and terminals." From observations on motor nerves, Eccles and Sherrington (1930) found that large fibres begin to branch more proximally as if to "...prepare early to form an extensive motor unit", and predicted that "...the cross-sectional area of the parent fibre gives an indication of the proportional size of the motor unit." The motor unit, which Liddell and Sherrington (1925) defined as "...an individual motor nerve-fibre with the bunch of muscle-fibres it activates", is an anatomical element which, when recruited into activity in combination with others, determines the final element of force in a given muscle. Henneman and his colleagues (Henneman et al., 1965a, 1965b) found that the diameter of a motor nerve fibre (as measured by axonal conduction velocity) is inversely related to the probability of motoneuronal discharge (ie, threshold for recruitment) during reflex evoked activation. These

observations led to the formulation of the size principle which predicts that motor units are recruited into activity in order of increasing size. An additional finding from these studies was a direct relation between nerve fibre diameter and force which suggested that larger axons do in fact supply more muscle fibres. While Henneman's size principle remains an important concept, the relative importance of motor unit size in determining its recruitment order is controversial. More recently, alternative mechanisms have been put forward to explain orderly recruitment and a variety of approaches have been taken to estimate motoneurone size (for more details see Kernell and Zwaagstra, 1981; Sypert and Munson, 1981; Burke et al., 1982; Zajac and Faden, 1985; Gustafsson and Pinter, 1985; Zengel et al., 1985; Pinter, 1990).

That large motor units produce large forces because they contain more muscle fibres is an intuitively attractive concept, but this is also controversial. As described by Burke (1981), the quantity of force developed by a motor unit is dependent on the product of three factors: 1) the number of muscle fibres innervated (innervation ratio, or IR), 2) mean muscle fibre cross-sectional area, and 3) the force generating capacity per unit cross-sectional area (specific tension). The relative importance of these three factors in determining force output is controversial since major discrepancies are found in the literature with respect to factors 1) and 3).

In normal muscle, motor unit force is well correlated with nerve fibre diameter and after reinnervation both the normal range of motor unit forces and the correlation between force and nerve fibre size is re-established (Gordon and Stein, 1982b). This suggests that the mechanisms determining motor unit force in normal and reinnervated motor units are similar. The present study was carried out to address this question with the underlying aim of resolving the controversy described above using both direct and indirect methods on the same population of motor units. A number of related questions were also addressed which will be introduced after a discussion of the background literature.

## 1.II Experimental Estimation of IR

Clark (1931) estimated the number of muscle fibres innervated by a motoneurone, the innervation ratio (IR), in the cat soleus muscle by counting the total number of motor axons and muscle fibres and found an average of 140-200 fibres per motoneurone. Using the same approach in human medial gastrocnemius (MG) muscle Feinstein et al. (1955) found an average IR of 1,730. Similar estimates of IR have been made in a number of different muscles since that time and the general finding is that IR increases as muscle size increases (see review by Buchthal and Schmalbruch, 1980). However, this approach using whole muscle data is inadequate to estimate differences in IR between individual

motor units. The work from Henneman's laboratory which showed that motor unit force and contractile speed varies over a wide range within the cat soleus and MG muscles and that nerve fibre diameter is related to force production (McPhedran et al., 1965; Wuerker et al., 1965) led the authors to suggest that the diameter of a motoneurone axon is related to the number of muscle fibres it supplies. The authors also suggested that there may be three kinds of motor units which correspond to the three kinds of muscle fibres observed histochemically in the MG (see Stein and Padykula, 1962 for more details on the histochemistry). Because of the overlap in the data however, only two types of units were distinguished in the MG: type A units were large (force producing), rapidly contracting and contained large axons, while type B units were smaller, slower and contained smaller axons. Although they did not attempt to determine the average IR for the different types, extrapolating from Clark's average IR value for the soleus muscle of 120 (which corresponds to an average force of 14.8 g) they estimated that the smallest (3.2 g) and largest (40.4 g) units should contain 39 and 491 muscle fibres, respectively. In the MG muscle, the smallest and largest IR values were 540 and 1,473, respectively. These estimates were based on the *a priori* assumption that fibre number does increase with force.

By stimulating motor units repetitively in the rat tibialis anterior (TA) muscle (10 Hz for 10 minutes) to induce the breakdown of fibre glycogen, Edstrom and Kugelberg (1968) were able to identify and enumerate individual muscle unit

fibres directly on cross-sections by their negative staining for glycogen. The results from their work showed that each muscle unit is essentially uniform in histochemical type and that the type A (type IIB with the nomenclature of Brooke and Kaiser, 1970) and B (IIA) units both contained an average of 132 fibres. Average twitch force in these same units was 1.8 g and 0.8 g. Since maximum (tetanic) force was not reported it is not known how it correlates with fibre number in these units. However, if one assumes a similar twitch-tetanic ratio for the two types of fast units, then fibre number apparently does not increase with force. An additional finding was that the succinate dehydrogenase activity (an oxidative enzyme marker) of each unit correlated well with its fatigability and with its histochemical type. This was demonstrated histochemically in this study but was verified quantitatively in a later study (Kugelberg and Lindegren, 1979). A few years later Brandstater and Lambert (1973) used the same glycogen depletion technique on the same muscle and reported a range of 55 to 172 muscle fibres per motoneurone (average of 118) but here again force was not reported. Burke and Tsairis (1973) used the glycogen depletion technique on cat MG units but stimulated at higher frequencies (40 Hz) for a longer time (15 to 60 minutes). They reported difficulty obtaining accurate fibre counts due to the fact that fibres in this muscle do not run end to end, but are relatively short and obliquely angled. This necessitates a complete proximo-distal reconstruction which was done for 3 units: 2 were classified as fast-twitch, fatigable (FF) and

1 was fast-twitch, fatigue resistant (FR) (see Burke et al., 1973 for the classification scheme). In this case fibre number does increase with force, and in conjunction with fibre area can account for the differences in force between these units. The smaller FF unit (35 g) contained 300 fibres while the larger one (120 g) contained 750 fibres. The FR unit developed force comparable to the smaller FF unit (39 g) but contained 1.7 times as many fibres (500) the mean area of which was 3 times smaller. Bodine et al. (1987) glycogen depleted 11 cat TA muscle units (40-70 Hz for 15-120 minutes) and for the first time obtained a relatively large sample of the three major motor unit types. Type S units contained significantly fewer fibres than the fast units but the overlap between FR and FF was extensive and IR was not significantly different. The data from all 11 units clearly showed a significant correlation ($r=0.94$) between force and IR.

The system of motor unit classification developed by Burke et al. (1973) which subdivided the MG motor unit population into three distinct groups: type S (slow contracting, fatigue resistant), FR (fast contracting, fatigue resistant), and FF (fast contracting, fatigue sensitive), provided the means to estimate IR indirectly for each type. By including fatigability into their classification scheme they were able to distinguish the three types that had previously eluded Wuerker et al. (1965) due to overlap in the data. In a previous report by Peter et al. (1972) where metabolic profiles of muscle fibres were examined, the three fibre types were described as slow-contracting-oxidative (SO), fast-contracting-oxidative-glycolytic

(FOG), and fast-contracting-glycolytic (FG). By obtaining the relative proportions of these three muscle fibre types ($p_i$) and the three different motor unit types ($q_i$) in the whole muscle, as well as the total number of muscle fibres (M) and motoneurones (N), Burke and Tsairis (1973) estimated the average IR for each type according to the equation (see Burke, 1981, p. 371):

$$IR_i = (M/N) \times (p_i/q_i) \quad (1)$$

and the value from the smallest S units (average force of 5.7 g) was 534 fibres, from the FR units (37.4 g) was 440 fibres and from the largest FF units (67.8 g) was 758 fibres. Although fibre number clearly increases with force when comparing the values from S and FF units, force is 12 times larger while IR is only 1.5 times larger for the FF units. When including muscle fibre area differences (FG 3 times bigger than SO) it is still not enough to explain these differences in force. Thus although the indirect estimates of IR agree reasonably well with the fiber counts from two of the three units (see above) it appears that averaging force and fibre areas from the whole population of like types obscures the differences within unit types. These results have since been confirmed by several different investigators in both flexor and extensor muscles (MG: Foehring et al., 1986, Gordon et al., 1988; flexor digitorum longus: Dum et al., 1985; tibialis posterior: McDonagh et al., 1980; and lateral gastrocnemius: Gillespie et al.,

1987) using the same indirect approach. Taken together, the indirect studies suggest that differences in motoneurone branching are small. The average number of fibres per motoneurone increases only 1.5 to 2-fold, mean fibre area increases 3-fold, while average force increases 10-fold or more between the smallest S and the largest FF units. This leaves specific tension to account for the rest of the variation in force. The validity of this approach of course, rests on the assumption that type S motor units consist of type SO muscle fibres and similarly FR=FOG and FF=FG and that the sampling of muscle fibre types and motor unit types is representative of the whole population.

## 1.III Estimates of Specific Tension

The specific tension of muscle is expressed as the total force developed per unit cross-sectional area. Estimates of specific tension have been made from a variety of different muscles from different species using both direct and indirect approaches. Direct estimates made by dividing muscle mass by fibre length (assuming a specific gravity of 1 g/ml) have indicated that fast twitch muscles have specific tensions about 1.5 times larger than slow twitch muscles (Barany and Close, 1971; Sexton and Gerston, 1967; Rowe, 1969; Kean et al., 1974; Julian et al., 1979; Edjtehadi and Lewis, 1979). In these studies however, fibre bundles from two different muscles have been compared using a 'predominantly'

fast muscle as a source of fast fibres and the 'predominantly' slow soleus muscle as a source of slow fibres. Furthermore, Close (1972) has suggested that these small differences between fast and slow muscles likely reflect differences in activation rather than specific tension. More recently, specific tension was measured from single skinned fast and slow fibres from the same muscle (Lucas et al., 1987). The values from fast (type II) and slow (type I) fibres were virtually identical indicating that fast and slow muscle fibres have the same capacity to generate force. The specific tension values from the glycogen depleted motor units of Bodine et al. (1987), obtained by dividing motor unit force by the directly measured total muscle unit cross-sectional area, were significantly larger for the fast units than for type S units. This difference was small however with fast units about 1.4 times larger than slow.

In contrast, the indirect method of estimating specific tension from motor unit data has indicated that type S motor units have specific tensions half that of FR or FF motor units (Burke and Tsairis, 1973; McDonagh et al., 1980; Dum et al., 1985; Foehring et al., 1986; Gordon et al., 1988). Values are again calculated by dividing motor unit force by the total muscle unit cross-sectional area but the latter term in this case is derived from the product of average IR and average muscle fibre size for a given type.

## 1.IV EMG and Motor Unit Size

As muscle force increases the electromyogram (EMG) signal also increases but the relation between these two parameters is complex. From studies on human gastrocnemius muscle Lippold (1952) found that integrated surface EMG increased linearly with force production. Clamann and Robinson (1985) found a non-linear relation between integrated EMG recorded intramuscularly and tetanic force in cat motor units such that the larger units produced relatively more electrical activity than small ones. From human first dorsal interosseus motor units, surface EMG amplitude was found to increase as the square root of twitch force (Milner-Brown and Stein, 1975). Theoretical studies using computer simulations have predicted that the root mean square EMG should increase as the square root of muscle fibre number (Moore, 1967). Whatever the exact relation between motor unit force and EMG is, the amplitude of the summed EMG potential should be in some way proportional to the the number of active muscle fibres (Katz, 1966; Kosarov et al., 1974). In the present study this could be directly tested in both normal and reinnervated motor units where the number of muscle fibres as well as their spatial distribution is known.

## 1.V Motoneurone Branching and Motor Unit Territory Size

Motor unit force is ultimately determined by the final number of motoneurone branches remaining after synapse elimination during postnatal development. Muscle fibres are initially innervated by a number of different motoneurones at the same synaptic sites and this polyneuronal innervation is gradually eliminated to result in a single synapse on each fibre (Redfern, 1970; Brown et al., 1976, Korneliussen and Jansen 1976). Thus motor units in the neonate are much larger than in the adult, estimated at 3 to 5 times larger (Brown et al., 1976; Thompson et al., 1984), and over the first few weeks of life the number of muscle fibres per motoneurone is dramatically reduced. This process of synapse elimination appears to involve competition between nerve terminals for the same endplate site as well as an inherent tendency for motoneurones to reduce the number of terminals (Thompson, 1986). Ultimately this process results in motor units each having a scattered distribution of muscle fibres which intermingle with those from other units. During reinnervation however, the patterns of branching are quite different as evidenced by the groupings of contiguous fibres within a muscle unit (Kugelberg et al., 1970), and the local competition for synapses during removal of polyneuronal innervation is also likely to be different. Recently, Landmesser et al. (1988) have found that axonal branching depends on the relative amounts of the cell adhesion molecules N-CAM (which promotes axon-myotube interactions)

and Ng-CAM (which makes axons adhere to one another). Since N-CAM is re-expressed in denervated muscle (Covault and Sanes, 1985) it is likely playing a role during reinnervation as well although the nature of adhesive interactions between regenerating nerves and denervated muscle is poorly understood. In spite of these differences in branching patterns during reinnervation, the same range of motor unit force is re-established, as well as the normal correlation between axon size and force (Gordon and Stein, 1982b; Gordon et al., 1986). If we consider also that fibre number is important in determining force (Bodine et al., 1987) this suggests that a property intrinsic to the motoneurone is controlling the number of branches it sustains and that this property is not lost after injury.

Examination of territory size, fibre density, and spatial distribution patterns from glycogen depleted muscle units may provide important information about branching patterns. For example, the distribution of reinnervated unit fibres may give some clues as to the possible role of intramuscular endoneurial tubes in the branching of regenerating motoneurones while muscle unit territory size is an indirect measure of the extent of axonal branching.

## 1.VI Summary of Objectives

The specific objectives in this study were:

1) to determine the relative contributions of innervation ratio, muscle fibre cross-sectional area, and specific tension to the observed variation in force between different motor units,

2) to determine whether these three factors determine force in the same way in reinnervated motor units,

3) to compare physiological and histochemical properties in normal and reinnervated muscle,

4) to compare direct and indirect methods of estimating innervation ratio on the same population of motor units and determine possible reasons for the previous discrepancies between the two methods,

5) to determine whether the number and spatial location of muscle fibres in normal muscle, and their altered spatial distribution in reinnervated muscle, influences the recorded EMG,

6) to determine how the magnitude of the EMG signal relates to other measures of motor unit size,

7) to examine the distribution patterns of fibres in muscle units from normal and reinnervated motor units,

8) to examine how muscle unit territory, an indirect measure of the extent of axonal branching, relates to motor unit force and whether this relation is altered after reinnervation.

# CHAPTER 2

# DIRECT MEASUREMENTS

## 2.I. INTRODUCTION

Nearly a century ago Ramon y Cajal (1909) concluded, from extensive histological examination of the nervous system, that large central axons branch more extensively than small ones. This general observation was extended by Eccles and Sherrington (1930) who reported that the larger $\alpha$-motoneurones begin to branch more proximally than the smaller ones. Henneman and his colleagues (Henneman et al., 1965a,b; McPhedran et al., 1965; Wuerker et al., 1965) later showed that motor units producing the smallest forces have the narrowest nerve fibre diameters (as indicated by slower conduction velocities) while large force producing units have larger nerve fibres. This result, in conjunction with the morphological observations described above, led to the hypothesis that large force producing motor units contain more muscle fibres. These and other size related properties of motor units have been incorporated into the **size principle** by Henneman (see review by Henneman and Mendell, 1981) which predicts a size-ordered recruitment of motor units during voluntary movement. That the size principle is of fundamental physiological significance is evidenced by the ordered recruitment of human motor units from small to large

during different types of voluntary movement (Milner-Brown et al., 1973; Desmedt and Godaux, 1977) and the re-establishment of the same size relationship between motor unit force and nerve fibre diameter as well as the same range of unit forces following recovery from nerve injury (Gordon and Stein, 1982b; Gordon et al., 1986; Foehring et al., 1986). While the size principle in its exquisite simplicity has provided a framework for the interdisciplinary approach to CNS control of muscle function, the importance of cell size in determining susceptibility to motoneuronal discharge, or recruitment order, (for more details see Kernell and Zwaagstra, 1981; Sypert and Munson, 1981; Enoka and Stuart, 1984; Gustafsson and Pinter, 1985; Zajac and Faden, 1985; Pinter, 1990) remains controversial. It is clear that the size of the motoneurone in some way influences the force of its muscle unit but it is not so clear how this is achieved.

As presented by Burke (1981), motor unit force production may be described by the following equation:

$$P_j = IR_j \times A_j \times ST_i \qquad (1)$$

where $P_j$ is the maximum tetanic force for an individual muscle unit, $IR_j$ is the unit innervation ratio, $A_j$ is the mean area of the constituent muscle fibres and $ST_i$ is the specific tension output (tension per unit cross-sectional area; see Close, 1972) of that muscle unit fibre type. There is conflicting evidence in the literature

on the degree to which each of these three variables influences the force output of a given motor unit. The role of specific tension is the most controversial since indirect estimates derived from motor unit and muscle unit properties indicate that it accounts for 50% (Dum et al., 1982), 60% (Burke and Tsairis, 1973) and up to 85% (Gordon et al., 1988) of the difference in force between unit types (see Stein et al., 1990, Figure 4). In contrast, recent direct measurements from single skinned fibres have shown no differences (Lucas et al., 1987) or very small differences (Laszewski, 1987) in specific tension between fast and slow types. Yet estimates derived from muscle fibre mass and length from whole muscle (Rowe, 1969) or isolated fibre bundles (Sexton and Gersten, 1967; Barany and Close, 1971; Kean et al., 1974; Edjtehadi and Lewis, 1979) have also indicated that fast muscle fibres have higher specific tensions than slow fibres. Additionally, a small but significant difference in specific tension was reported between fast and slow cat tibialis anterior motor units (Bodine et al., 1987). Indirect estimates of IR from the proportion of of muscle fibre types to the proportion of motor unit types indicate that there is little difference in the number of muscle fibres contained in S, FR, FI, and FF motor units, which was supported by data from three depleted units from cat MG muscles (Burke and Tsairis, 1973). These indirect data, together with relatively small differences in fibre area between types, led to the conclusion that IR was a minor factor in determining unit force. $ST_i$ and $A_j$ were the major factors which accounted for the 100-fold difference in

force capacity of motor unit types where S < FR < FI = FF. Re-examiniation of IR using glycogen depletion to directly visualize muscle fibres in isolated and characterized motor units, indicated that $IR_j$ is a significant contributing factor to normal motor unit force (Bodine et al., 1987). Thus, the relative importance of fibre number remains controversial.

Following nerve injury and either self- or cross-reinnervation of denervated muscles, reinnervated motor unit force recovers to preoperative levels and unit force is well correlated with electrophysiological measures of nerve fibre diameter (Gordon and Stein, 1982a,b; Gordon et al., 1986; Foehring et al., 1986). Since regenerating nerves do not reinnervate their original muscle fibres, even after self-reinnervation, restoration of the normal range of motor unit force and the normal size principle implies considerable adjustment of unit properties. This raises the question whether the same mechanisms are operating in normal as in reinnervated muscles to determine force output. To what extent is it alteration in muscle properties ($ST_i$ and $A_j$) that accounts for recovery of the size principle and is the extent of branching a relatively random process? The altered spatial distribution of muscle fibres (Karpati and Engel, 1968; Kugelberg et al., 1970) and lack of specificity of nerve fibres for their original muscles (Gillespie et al., 1987) suggests that reinnervation is a relatively non-specific process, in contrast to the ordered branching patterns observed during development (Landmesser, 1984).

The present study was undertaken to address the question of the relative contribution of motoneuronal branching (IR) and muscle factors (ST and A) to the normal force development of motor units and whether these contributions are different in reinnervated muscle after injury. Using glycogen depletion to identify muscle units in normal and reinnervated motor units in rat tibialis anterior (TA) muscles, our findings indicate that IR is a significant contributing factor to force development in both normal and reinnervated motor units and that specific tension is not a systematic deteminant of force. A preliminary account of the results has previously been published in abstract form (Totosy de Zepetnek et al., 1988).

## 2.II. METHODS

### 2.II.1 Experimental Denervation

Young female Sprague Dawley rats weighing 180-200 grams were anaesthetized with an intraperitoneal (ip) injection of 45mg/kg sodium pentobarbital (somnotol). Under aseptic conditions, the common peroneal nerve was isolated in either the right or left hindlimb. A fine glass rod was placed under the nerve and a 6-0 or 8-0 suture was used to draw thread through the nerve in a loop around the glass rod. The end of the thread was tagged with a haemostat and the glass rod was used to pull the thread away from the nerve and make it slack. Taking care not to cut the thread, the nerve was transected, and the proximal and distal stumps were reconnected using the thread already in place. This strategy made the surgery quick as well as successful in promoting good reinnervation of the hindlimb flexor muscles. A total of 40 rats received this surgical treatment and were used for the final acute experiment 3.5 to 8 months after the initial surgery.

### 2.II.2 Surgical Procedure for Acute Experiments

Female Sprague Dawley rats (220-420 g) prefed for 3-5 days with 5% glucose in the drinking water were anaesthetized as above and 0.1 mg/kg atropine sulphate was administered i.p. to reduce tracheal secretions. The trachea was cannulated so that mechanical ventillation could be performed if necessary and the right external jugular vein was cannulated for intravenous administration of maintenance doses of anaesthetic. The anaesthetic was diluted 1 in 5 with a 5% dextrose-saline solution and administered for maintenance anaesthesia approximately every 1-2 hours. Spinal roots L1 to L6 were exposed by laminectomy and all hip, tail and hindlimb muscles were denervated with the exception of the TA, as illustrated in Figure 2.1A.

The bared ends (approximately 5 mm) of two teflon coated fine silver wires (75 $\mu$m) were inserted into the adductor femoris and semi-membranosus muscles approximately 10 mm apart longitudinally on either side of the sciatic nerve to serve as stimulating (indirect) electrodes. This was done by threading the wire through a 27 gauge needle and wrapping the bared end around the needle to produce a coil. The needle was then introduced into the underlying muscle and withdrawn, leaving the coiled wire within the muscle. It was found that the coil was more effective than a simple hook in delivering the stimulus to the nerve.

The TA muscle was dissected free from surrounding tissue over its distal half, taking care not to disturb its innervation or blood supply. A bipolar electromyogram (EMG) probe was sewn onto the fascia over the middle third of the muscle and served as both a stimulating (direct) and a recording electrode. The distal tendon of the TA was freed along with a small piece of calcaneous bone around its point of insertion and securely tied with 2-0 silk suture. To monitor muscle temperature, a temperature probe was sewn onto the musculature above the knee. Finally, skin along the incision line was reattached using metal clips.

Both hindlimbs were immobilized at the knees and ankles with clamps and the distal TA tendon in one leg was secured to a 1 kg Grass force transducer. Care was taken to maintain the in vivo position of the muscle. To make a mineral oil pool around the spinal cord, rubber bands were clipped to the skin flaps and attached to clamps above the animal to extend the skin. Temperatures were monitored from the lower hindlimb muscles, the spinal cord and the rectum with temperature probes (Yellow Springs Instrument Co.), and maintained between 35° - 37°C by radiant heat above and a heating pad underneath the animal. After the spinal cord was immersed in oil, with the aid of a microscope an incision was made along the dura mater to free the spinal roots. Ventral roots L3, L4 and L5 were isolated using gentle suction and cut as far up rostrally as the laminectomy would allow.

### 2.II.3 Total Muscle Force

The optimal length of the muscle for maximum force development was determined by recording twitch force in response to suprathreshold single stimulus pulses delivered through the EMG probe and, with a micromanipulator, progressively stretching the muscle from its initial slack length to optimum length. Stimulus pulses were also delivered to the muscle through the sciatic nerve for a comparison of responses to direct and indirect stimulation. Force and EMG signals were amplified, displayed with a Tektronix dual time base storage oscilloscope as well as with a Gould 1200S pen recorder, and digitized and stored on disk by an LSI-11 computer (Digital Equipment Corporation).

### 2.II.4 Motor Unit Isolation and Characterization

Single motor units from the TA muscle were functionally isolated by stimulating fine filaments dissected from one of ventral roots L3, L4, or L5 (Figure 1A). The criteria for single unit isolation were a) the signals were clearly "all or none" to threshold stimulation and b) over several minutes there was no change in the force or EMG recordings. A number of physiological properties of the single unit were measured for characterization as slow-contracting (S), fast-contracting fatigue resistant (FR), fast-contracting fatigue intermediate (FI), and

fast-contracting fatigable (FF). The computer was used to average a number of EMG and force signals for each stimulus pattern:

**2.II.4(a) Twitches**

For each twitch response the following parameters were measured: peak force in mN, time to peak force (contraction time), half rise time, and half fall time all in ms as shown in Figure 2.1 C. 5 - 10 twitches at 1 Hz were averaged from each unit.

**2.II.4(b) Tetanus**

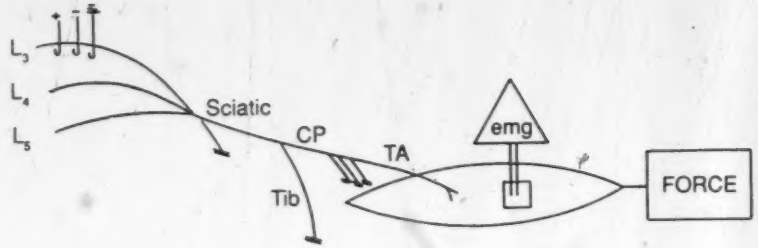
Previous work from this laboratory on rat muscle (Gillespie et al., 1987) has shown that tetanic force is maximal with 21 to 30 pulses at an inter-pulse interval equivalent to one-third the twitch contraction time. 3-5 tetanic trains at 1 Hz were averaged by the computer and peak force, half rise and half fall times were measured (Figure 2 D).

**2.II.4(c) Sag Tests**

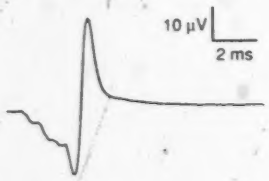
The sag test developed by Burke et al. (1973) has been used to subdivide fast (F) from slow (S) motor units. Peak force during an unfused tetanus (interpulse interval (ipi) 1.25 times twitch contraction time; 800 ms long) declines or 'sags' in F units while in S units force progressively rises. In this study the first response to such a stimulus was recorded as well as the average of six responses (called sag1 and sag6, respectively; see Figure 2.1 G for an example of a fast contracting unit exhibiting sag). Responses to two other stimulus frequencies modified from Larsson and Edstrom (1987) were measured: 200

Figure 2.1. Diagram showing isolation of a single motor unit (A) and the physiological parameters measured (B - G). EMG signal (B) amplitude is measured from the negative to the positive peak in microvolts. Peak force of the twitch (C) is measured in millinewtons (mN), and time to peak force (TTP, a), half contraction time (1/2 CT, b), and half fall time (1/2 FT, c) are measured in milliseconds (ms). Time to peak force is measured from the point at which force just begins to increase above zero to the point where force first reaches maximum. The progressive fatigue test (13 stimulus pulses at 40 Hz, 1/sec for 2 minutes; see Burke et al., 1973) is shown in its entirety (E) as well as individual contractions at 0, 1 and 2 minutes of the test (F). The ratio of peak force at 2 minutes to that at the start of the test (time 0) is widely used to classify motor units into physiological types (see text for more details). The ability to maintain force during an unfused tetanus (800 ms stimulus at an ipi 1.25 times CT), is characteristic of slow contracting motor units while the fast contracting units normally show a decline, or sag, in force (G).

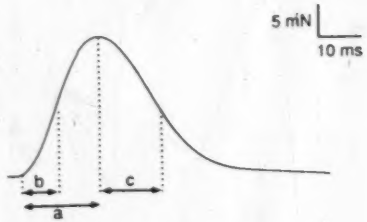
## A. ISOLATION OF A SINGLE MOTOR UNIT




## B. EMG

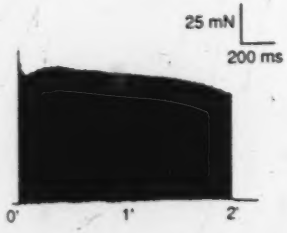



## C. TWITCH

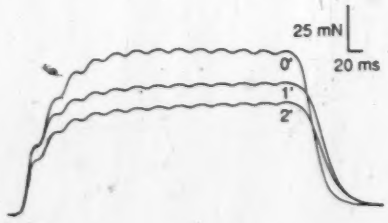



## D. TETANUS

## E. PROGRESSIVE FATIGUE TEST




## F. FATIGUE




## G. SAG

pulses at 50 Hz (sag200) and 800 pulses at 200 Hz (sag800) - both lasting a total of 4 seconds. An index of sag was generated from each of these tests from the quotient of final force to peak force (see Results).

**2.II.4(d) Fatigue Test**

Following the sag test the unit was stimulated with 13 pulses at 40 Hz delivered once per second for 2 minutes as shown in Figure 1E and F (see also Burke et al., 1973). The fatigue index is the ratio of the force developed during the last contraction in the series to that of the first. Units were classified as S if the contraction time exceeded 22 ms while the faster contracting units were subdivided according to their fatigue index: FR > 0.75; 0.75 > FI > 0.25; and FF < 0.25.

**2.II.5 Glycogen Depletion**

Once the motor unit was completely characterized, the muscle unit was depleted of its glycogen for later histochemical identification. The way in which the unit was stimulated to result in glycogen depletion depended on its physiological properties and a modification of the technique of Kugelberg and Lindegren (1979) was used as follows:

**2.II.5(a) Fast-contracting motor units**

For each fast unit a minimum of five successive stimulation-recovery cycles were carried out. The stimulus pattern began with 21 pulse 100 Hz trains delivered at a duty cycle of 1 Hz. If after several minutes there was no perceptible decline in force production and the EMG signal remained stable, the duty cycle length was progressively decreased until force began to decline. When force declined to less than 10% of the initial level, the recovery period was initiated. If at any time the EMG signal showed signs of decline (e.g. some pulses in the train not getting through to the muscle) either the number of pulses per train was decreased or the recovery cycle initiated. The recovery stimulus consisted of 21 pulse 100 Hz trains at a duty cycle of 0.1 Hz and was continued until the force increased to a stable level whereupon the depleting stimulation recommenced. For the more fatigue resistant units which generally take longer to break down their glycogen stores, brief periods of ischemia (15-20 seconds) were induced by compressing the femoral artery. This was repeated as many times as necessary to reduce force to near zero at the same time ensuring that the EMG signal was well maintained.

**2.II.5(b) Slow-contracting motor units**

For the slow units a different approach was taken because of their high resistance to fatigue as well as to glycogen depletion (Edstrom and Kugelberg, 1968; Burke and Tsairis, 1973). Stimulation consisted of trains of 21 pulses at

100 Hz (at the same rate of 1/sec as above) delivered for up to 2 1/2 hours. After 2 hours of stimulation brief periods of ischemia were induced as described above in order to force the muscle unit to use its stored glycogen to maintain contractile force. As long as the units were stable enough to carry out this entire procedure, it resulted in well depleted slow units.

Upon cessation of stimulation the TA muscles from both legs were very quickly excised and placed on saline-soaked gauze squares on ice. Muscles were weighed, cut into three blocks, and each block was mounted with OCT (ornithine carbamyl-transferase) compound onto a cork, frozen in a pool of melted isopentane cooled with liquid nitrogen (-160°C) and immediately placed in a freezer (-90°C) for storage.

### 2.II.6 Histochemistry

Serial cross-sections 10 $\mu$m thick were cut from each muscle block in a cryostat at -20°C. The following histochemical stains were used: myofibrillar ATPase with acid preincubation modified from Brooke and Kaiser (1970), myofibrillar ATPase with alkaline preincubation modified from Guth and Samaha (1970), periodic acid-Schiff (PAS) according to Pearse (1960), and nicotinamine adenine dinucleotide diaphorase (NADH) according to Dubowitz and Brooke (1973). The PAS staining was used to identify and enumerate muscle fibres

belonging to the previously stimulated motor unit while the remaining stains were used for histochemical typing and are described in more detail in Chapter 3.

### 2.II.7 Muscle Unit Fibre Counts and Area Measurements

Camera lucida drawings were made from a series of PAS-stained sections taken from different proximodistal levels of the muscle. Each fibre of the glycogen depleted muscle unit was recorded on the drawing which was then superimposed with serial sections stained for myosin ATPase for type identification. Glycogen depletion was verified in all fibres under high power (25X) magnification by the absence of reticulum. In this way the muscle unit was directly enumerated and typed at several levels along the muscle length. The section containing the greatest number of fibres was used to measure fibre cross-sectional areas. In the initial experiments, cross-sectional areas were measured of all fibres of the muscle unit from photomicrographs by planimetry using a digitizing tablet (Summagraphics MM1200 series). In later experiments the PSICOM 327 Computer Image Processing System was used. The image processor consists of a light microscope (Zeiss) connected to a video camera (Perceptive Systems Inc., Model NC66LX) which sends a live image of the magnified muscle section to a video display monitor. From the live image, the PSIEXEC software (version 2.4) could produce a grey level image by means of

picture elements (pixels), previously calibrated for optical density, ranging from 0 (black) to 255 (white). Those cells depleted of glycogen consistently had grey level values well above 200 while the remaining cells were well below 200 and a binary image was obtained by choosing a cut-off value between the two. In the final image (after editing the unwanted features such as tendon, etc) only the depleted cells were present and their cross-sectional areas could be directly measured with the software. When both techniques were used (visual identification of the unit and area measurements from photomicrographs and unit identification and area measurement with the image processor) the same results were obtained. Thus, the 'manual' technique works as well as the image processing system for unit fibre number enumeration, but it takes considerably more time.

### 2.II.8 Statistics

Standard techniques were used to compute arithmetic and geometric means plus standard errors. To compare values from normal and reinnervated populations, either the t-test or the Kolmogorov-Smirnov test (Fisz, 1963) was used. Data were fitted with straight lines according to the least mean squares criterion of Sokolnikoff and Redheffer (1958).

## 2.III. RESULTS

### 2.III.1 Glycogen Depletion

Records of force output and EMG during repetitive tetanic stimulation to produce glycogen depletion are shown in Figure 2.2 and are representative of a slow contracting fatigue resistant unit, a fast contracting fatigue resistant and a fast contracting fatigue sensitive unit. Prior to glycogen depletion, all units were completely characterized physiologically and these results are presented in Chapter 3. The slow contracting unit in A was stimulated continuously for 2 hours after which time the femoral artery was briefly occluded to induce ischemia. Approximately 15 seconds of ischemia, repeated a number of times, was extremely effective in reducing the force generating capacity of fatigue resistant units and resulted in complete depletion of glycogen in that muscle unit. The depleted muscle unit from this experiment is shown in cross-section in Figure 2.3 and it is clearly type I (or SO). Early in this series of experiments we found that prolonged ischemia, which rapidly reduces force, often led to muscle damage which obscured identification of depleted muscle fibres or to loss of the unit during recording. On the other hand brief periods of ischemia reduced force, and presumably glycogen, more slowly, allowed good recovery of EMG and force during the recovery periods and maintained the health of the tissue.

Figure 2.2. Records of glycogen depletion of fatigue resistant and fatigue sensitive motor units. The slow unit in A was stimulated continuously with 21 pulses at 100 Hz for 2 hours (duty cycle of 1 Hz), after which time the femoral artery was repeatedly occluded to induce ischemia. The arrows indicate the duration of ischemia and shown here are the 7th and 8th ischemic trials. This resulted in a dramatic decline in force production and effectively depleted fibre glycogen (see Figure 2.3). Note the stability of both force and EMG during the 2 hours of stimulation. In B a fatigue intermediate unit is shown which received intermittent stimulation, ischemia (marked by the arrows), and recovery periods (21 pulses at 0.1 Hz). The star indicates an increase in the duty cycle frequency from 1/sec to 1/800 ms. The fatigue sensitive unit in C shows a rapid reduction in force within the first minute of stimulation. Note that the time scales are different in different sections of the figure.

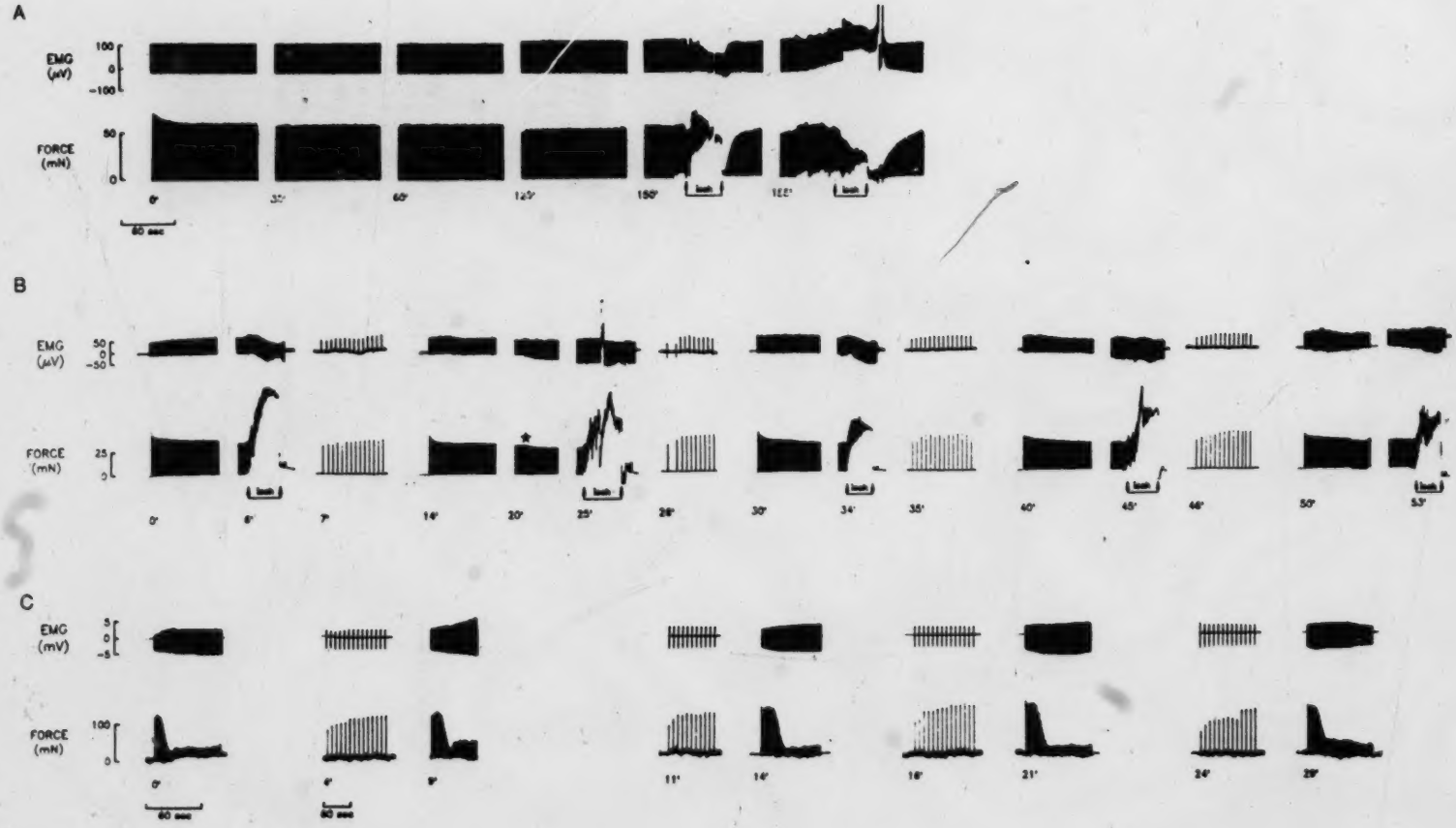
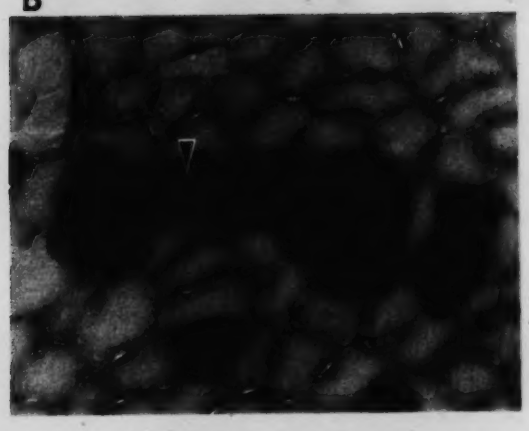

A
EMG (μV)
100
0
−100
FORCE (mN)
50
0
60 sec
B
EMG (μV)
50
0
−50
FORCE (mN)
25
0
C
EMG (mV)
5
0
−5
FORCE (mN)
100
0
60 sec

Figure 2.3. Serial sections from a reinnervated muscle containing a glycogen depleted slow muscle unit. The section stained with PAS (A) shows a group of negative staining fibres which, on the serial sections stained for myosin ATPase with acid preincubation (B) are dark and with alkaline preincubation (C) are light indicating a well depleted reinnervated slow muscle unit.

A

B



C

The fast contracting units showing resistance to fatigue required longer stimulation than the fast fatigue sensitive units but considerably less than the slow contracting units for successful glycogen depletion (Figure 2.2 B). Brief periods of ischemia were often used to further reduce force production and enhance the process of depletion. Increasing cycle frequency partway through the protocol proved to be a successful approach in both fatigue resistant and fatigue intermediate units. Fast contracting fatigue sensitive units, an example is shown in Figure 2.2 C, were considerably easier to deplete and required relatively brief periods of fatiguing stimulation with recovery periods in between. The EMG signal was generally less stable in these highly fatigable units suggesting possible neuromuscular fatigue but as previously noted by Edstrom and Kugelberg (1968) this did not prevent the muscle unit from becoming completely depleted of glycogen. In cases where the EMG signal showed signs of decline towards the end of the stimulus trains, one of two courses of action was taken: 1) If it occurred near the beginning of the protocol, the fatiguing stimulation was stopped and recovery was initiated regardless of how far force had declined. 2) If it occurred in the middle or near the end of the stimulus regime the pulse number was decreased and cycle frequency increased to maintain a high work to rest ratio. The rationale behind 1) was based on preliminary experiments where we found that if the EMG signal declined early in the stimulus regime, decreasing pulse number and increasing cycle frequency

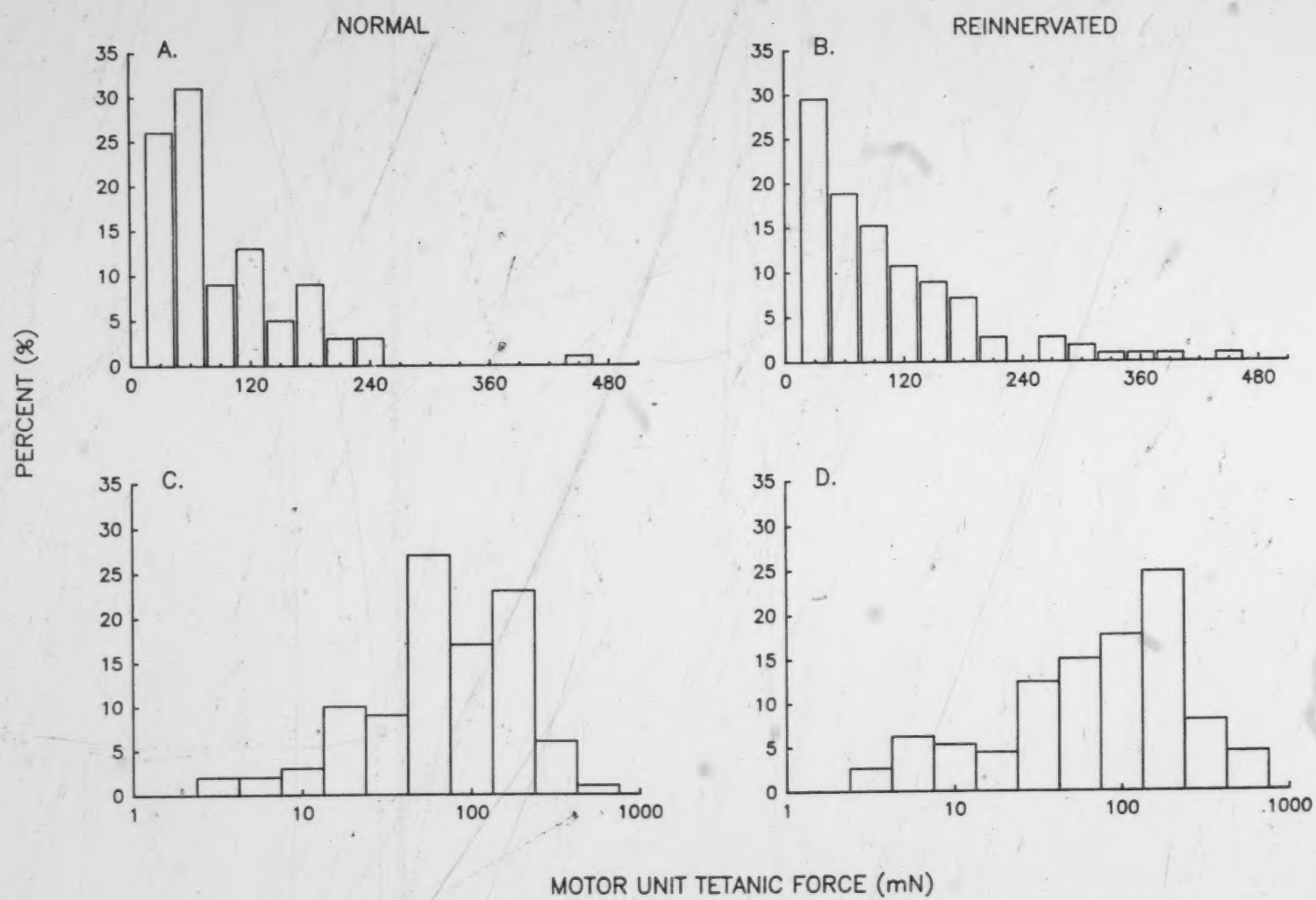
increased the likelihood of losing these fatigue sensitive units entirely.

Out of 29 normal and 32 reinnervated motor units for which this protocol was applied, 20 muscle units of each were successfully depleted. Failures in depletion were generally attributable to either loss of the unit during the stimulation period, or generalized muscle damage due to freezing artifacts or prolonged ischemia. However, 3 normal and 5 reinnervated units were incompletely depleted due to their instability which prevented them from being optimally stimulated. In these muscles there was a population of fibres detectably paler than the rest on PAS however these cells still contained a small amount of glycogen (they were slightly pink) and for this reason were not included in the final analysis. No differences were found between normal and reinnervated units in their susceptibilty to glycogen depletion.

### 2.III.2 Distribution of Motor Unit Force

The distribution of tetanic force is markedly skewed to the right on a linear scale indicating a much larger proportion of small force producing units in both normal and reinnervated muscle (Figure 2.4 A and B). On a logarithmic scale the distributions are much less skewed (C and D). This has been reported previously for a number of cat and rat hindlimb muscles (Burke et al., 1973; Dum and Kennedy, 1980; Gordon and Stein, 1982a). Note that the normal and reinnervated

Figure 2.4. Distribution of tetanic force from normal and reinnervated motor units. In A and B the scale is linear showing the skewed distribution towards smaller forces in both normal and reinnervated motor units. In C and D the scale is logarithmic and the distributions are much less skewed.


NORMAL
REINNERVATED
A.
B.
C.
D.
PERCENT (%)
MOTOR UNIT TETANIC FORCE (mN)
0
5
10
15
20
25
30
35
120
240
360
480
1
10
100
1000

units develop force over a similar 100-fold range. The cumulative distributions from normal and reinnervated muscle in Figure 2.5 are not significantly different and show that the proportion of units at each force value is the same in both groups.

From the total population of sampled motor units there were 20 normal and 20 reinnervated units that were depleted of glycogen and could be used for direct counts of muscle fibre number. When the glycogen depleted units are considered separately in Figure 2.6 it is evident that the range of force developed by these units is similar to that of the whole population. The cumulative distributions in C and D show that the proportion of depleted units at each force level is the same as that of the whole population thus the total population is well represented by the depleted units. It is important to note that the range of tetanic force developed by the whole muscle is also similar between the two groups with mean values (± standard error) of 6471.6 ± 312.0 and 6236.5 ± 265.9 mN for control and reinnervated, respectively, indicating that reinnervation of these muscles was highly successful.

Figure 2.5. Cumulative distributions of tetanic force from normal and reinnervated motor units. Cumulative distributions on a logarithmic scale can readily show differences between populations if there is a parallel shift or a change in the shape of the curve. The distributions from normal and reinnervated muscle are virtually superimposable however and the Kolmogorov-Smirnov test (Fisz, 1963) showed no significant difference between them ($P > 0.05$).

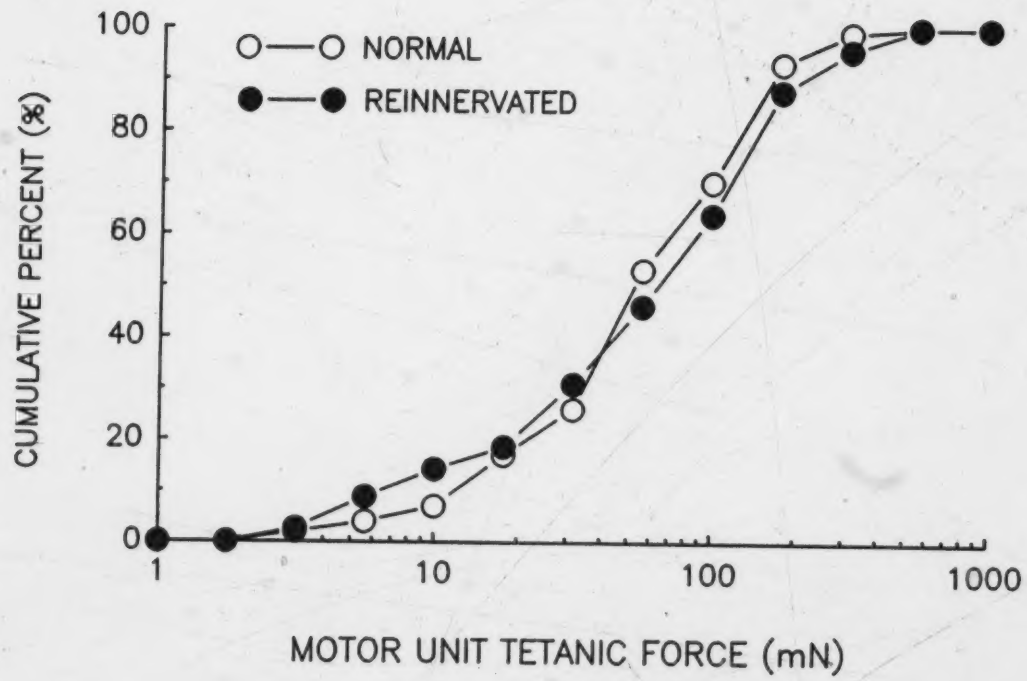
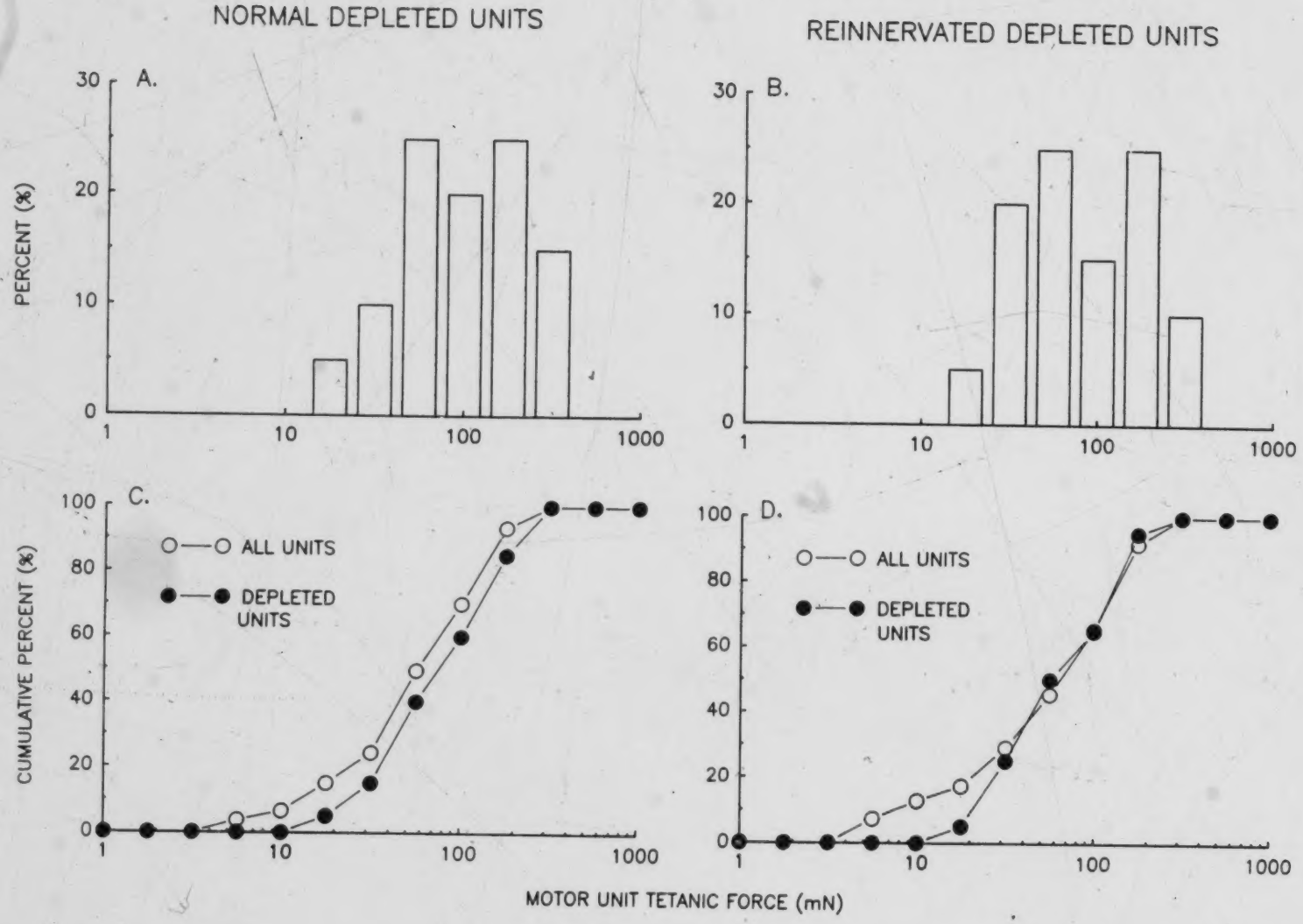

100
80
60
40
20
0
CUMULATIVE PERCENT (%)
NORMAL
REINNERVATED
1
10
100
1000
MOTOR UNIT TETANIC FORCE (mN)

Figure 2.6. Distribution of tetanic force from the glycogen depleted normal and reinnervated motor units. A (normal) and B (reinnervated) show the range of force developed by the depleted units is similar to that of the whole population of sampled units (see Figure 2.4). The cumulative distributions in C and D show that the proportion of depleted units at each force level is not significantly different from that in the whole population (Kolmogorov-Smirnov test, $P > 0.05$; Fisz, 1963).


NORMAL DEPLETED UNITS
REINNERVATED DEPLETED UNITS
A.
B.
C.
D.
PERCENT (%)
CUMULATIVE PERCENT (%)
ALL UNITS
DEPLETED UNITS
MOTOR UNIT TETANIC FORCE (mN)

### 2.III.3 Identification of the Muscle Unit

Muscle cross-sections stained for periodic acid-Schiff (PAS) allow the glycogen depleted muscle fibres to be identified by their negative staining. The dramatic contrast in PAS staining between the stimulated and unstimulated fibres allowed enumeration and cross-sectional area measurements of all the constituent fibres of the muscle unit. Histological examination of reinnervated muscle cross-sections showed these muscles to be healthy with no evidence of muscle fibre atrophy. A typical distribution pattern of muscle fibres from a normal unit is shown in Figure 2.7 A. The fibres are widely scattered covering a relatively large region of the muscle cross-section whereas the reinnervated unit in B is highly clumped and covers a smaller territory.

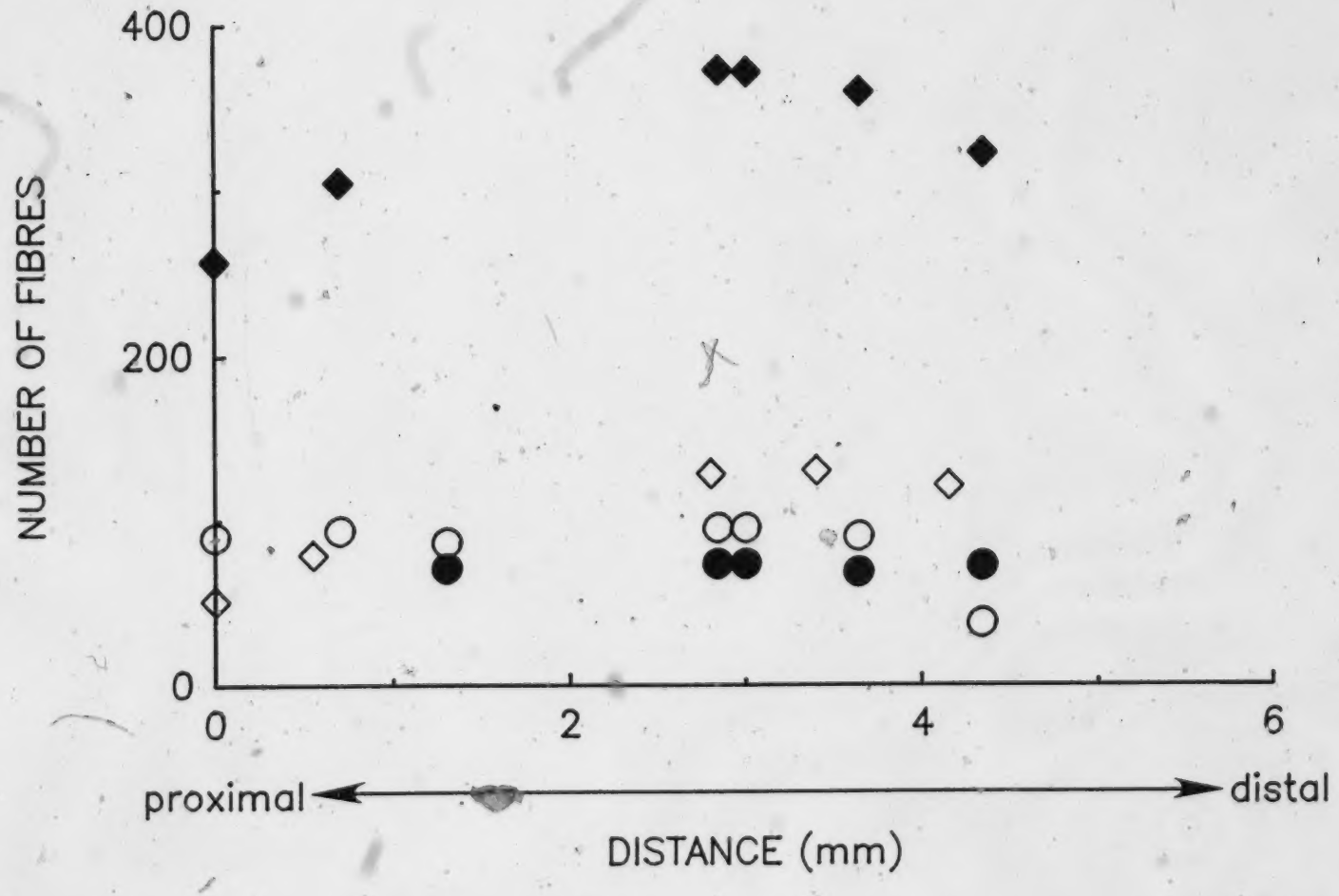
Muscle unit fibre counts were initially made from a number of sections from different proximodistal levels of the muscle to assess the variability in these counts over fibre length. The maximum number of muscle unit fibres was found in the middle third or mid-belly, which corresponds to its thickest part (Figure 2.8). Within the middle region the variation in fibre counts from sections hundreds of microns away was small (0.6 - 4.4 percent) and the maximum count from this region was taken as a reasonable estimate of total muscle unit fibre number. This small variability in fibre counts from different muscle regions agrees well with what has been reported from similar direct measurements of cat TA muscle units (Bodine et al., 1987).

Figure 2.7. Photographs of PAS-stained cross-sections showing the muscle fibre distribution from a normal and reinnervated glycogen depleted muscle unit. The normal muscle unit (A) has a scattered distribution extending over a relatively large territory while the reinnervated unit (B) is highly clumped and occupies a smaller territory.

A

B

Figure 2.8. Motor unit fibre counts taken from different proximodistal levels of the muscle. The number of glycogen depleted fibres from two normal (open symbols) and two reinnervated (filled symbols) muscle units are plotted as a function of distance along a proximodistal gradient. Proir to freezing, muscles were cut into three blocks. The set of points from 0 to 1.3 mm were taken from the most proximal block and zero represents the level of the muscle from which the first sections were taken. Over the distances from 2.8 to 4.4 mm, which corresponds to the middle block at the muscle mid-belly, fibre number remains relatively constant.


400
200
0
NUMBER OF FIBRES
0
2
4
6
proximal
distal
DISTANCE (mm)

The depleted fibres showed small shifts in position over distances of about 200 microns but neither the total fibre counts nor the territory of the muscle unit changed. From the sections examined from the mid-belly, the one yielding the highest count was selected for further analysis. This involved type identification and cross-sectional area measurement of each depleted cell as well as spatial or territorial analysis of the entire muscle unit. The latter analysis is discussed in Chapter 6. Histochemical typing of depleted fibres from serial sections stained for myosin ATPase (alkaline and acid preincubation - see methods) and NADH revealed that the fibres comprising both normal and reinnervated muscle units are essentially histochemically uniform (see Chapter 3) which is consistent with previous reports (Edstrom and Kugelberg, 1968; Kugelberg et al, 1970; Burke and Tsairis, 1973).

### 2.III.4 Determinants of Motor Unit Force

As shown by equation 1, motor unit force depends on the number of muscle fibres innervated, their mean cross-sectional area, and their specific tension. In a previous report (Stein et al., 1990) it was shown that by taking the logarithm of equation 1, each of the three factors become additive and it is then possible to estimate their relative importance in determining motor unit force output:

$$\log P_j = \log IR_j + \log A_j + \log ST_j \quad (2)$$

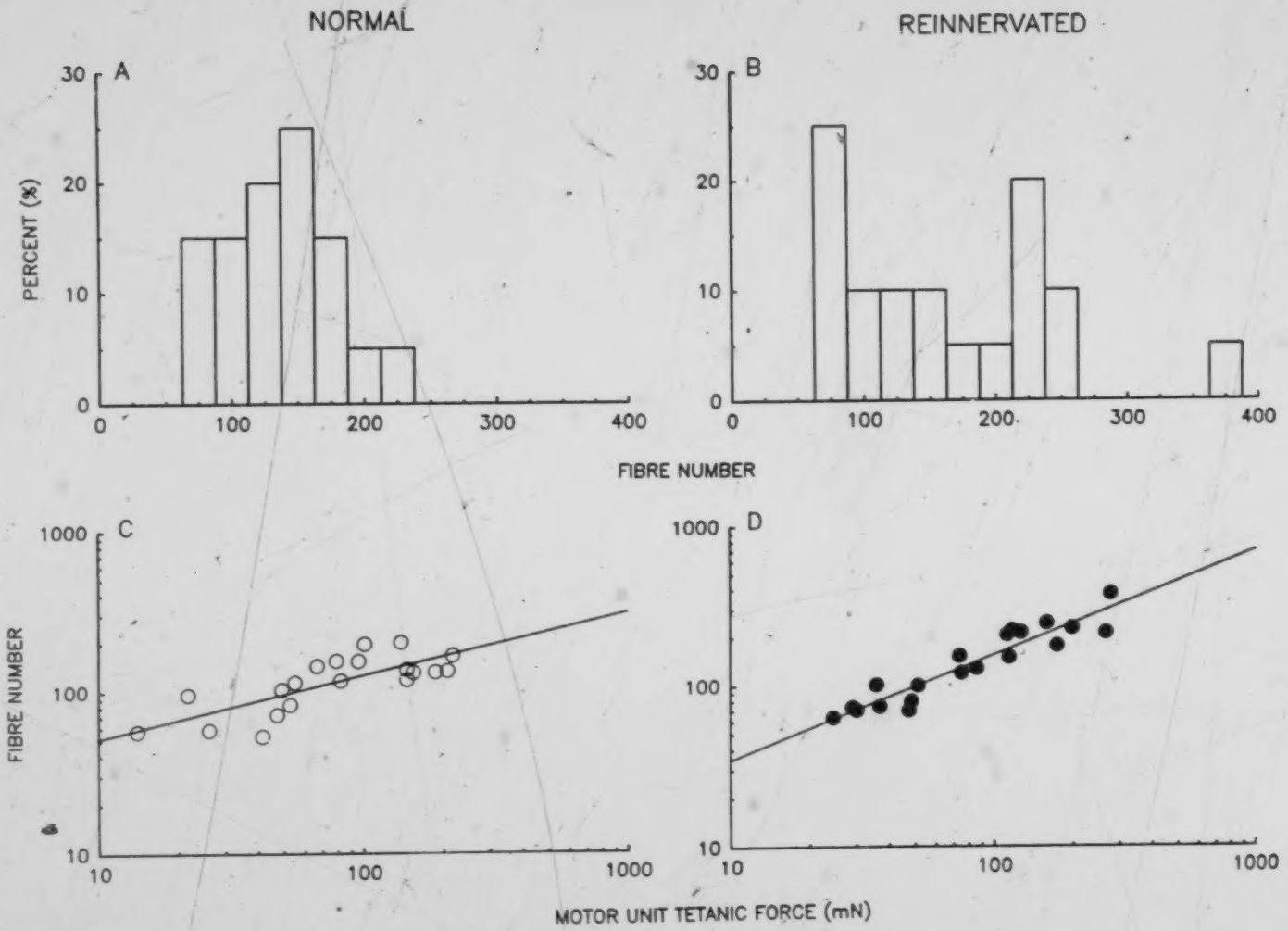
When each factor is plotted on a double logarithmic scale against force ($P_j$), the slope of the line gives the relative contribution of that factor to the observed variation in force. The contributions of each of these factors to force in normal and reinnervated TA muscles is considered in turn below.

**2.III.4(a) Motor Unit Fibre Number (Innervation Ratios)**

Considering the maximum muscle fibre counts as a measure of muscle unit size, it is evident from Figure 2.9 A that the sizes in normal muscle vary over approximately a 3.5-fold range. The size range of reinnervated muscle units is similar, with the exception of one unit which was considerably larger than the rest.

Mean unit fibre number was 121.2 ± 9.4 and 153.1 ± 17.7 for normal and reinnervated, respectively, which compares favourably with the measurements from Kugelberg's laboratory of 127 in normal and 145 in reinnervated rat TA units (Edstrom and Kugelberg, 1968; Kugelberg et al., 1970) and with Brandstater and Lambert (1973) of 118 in normal TA units. Fibre number in normal muscle increases with unit tetanic force (C) which shows directly that, as predicted, the number of muscle fibres is an important determinant of motor unit force. This is consistent with results from the previous direct study by Bodine et al., (1987).

Figure 2.9. Distribution of muscle fibre number and the relation between motor unit tetanic force and muscle fibre number. The distributions of muscle fibre number for 20 normal (A) and 20 reinnervated (B) glycogen depleted motor units show a greater concentration at the two ends of the scale in reinnervated units. C and D show that fibre number varies positively with tetanic force and the regression lines have a slope ± standard error of 0.39 ± 0.08 for normal and 0.65 ± 0.06 for reinnervated units. The correlation coefficient is lower in the normal plot, 0.77, compared to the reinnervated value of 0.94.


NORMAL
REINNERVATED
A
B
C
D
PERCENT (%)
FIBRE NUMBER
FIBRE NUMBER
MOTOR UNIT TETANIC FORCE (mN)
0
10
20
30
100
200
300
400
1000

The present results show further that fibre number is again related to unit force in reinnervated units (D) and in fact becomes an even larger contributing factor to unit force than in normal muscle. The slope of the log-log plots indicate that fibre number accounts for 39% of the variation in force in normal muscle and 65% in reinnervated muscle. Bodine et al. (1987) found, using a step-wise regression analysis, that fibre number accounted for 88% of the variation in force in normal cat TA. If the data from this study is re-expressed using the logarithm of equation 1, the relative contribution of fibre number becomes 64% - a value similar to what we have found in reinnervated rat TA.

Since there is clearly a tendency for larger force producing units to contain more muscle fibres, and differences exist between physiological types with respect to force output, it follows that fibre number will differ between types as is shown in Table 2.1 A. Mean force and mean fibre number also differ between unit types in reinnervated muscle (Table 2.1 B). Note that the reinnervated SO units are about twice as large as normal with respect to mean force output but that mean fibre number increased by only 1.6, concordant with the increased size of the muscle fibres. The mean number of fibres was significantly higher in all fibre types in reinnervated muscle except in FR units. The ratio of fibre number to force is similar but the overlap between unit types is greater as evidenced by the high standard error of the mean. Note that the type distribution was significantly altered from a predominantly fatigable to less fatigable muscle.

Table 2.1 SUMMARY OF THE GLYCOGEN DEPLETED MOTOR UNIT DATA

A. NORMAL

| | FORCE[a] | FIBRE NUMBER | MEAN AREA[b] | SPECIFIC TENSION[c] |
|---|---|---|---|---|
| SO | 14.0 | 57 | 1135 ± 45 | 2.20 |
| | 26.1 | 58 | 1499 ± 53 | 3.06 |
| FOG | 21.7 | 96 | 1190 ± 17 | 1.94 |
| | 82.2 | 117 | 2906 ± 67 | 2.47 |
| FInt | 49.2 | 103 | 1878 ± 37 | 2.59 |
| | 53.0 | 83 | 4142 ± 136 | 1.57 |
| | 79.2 | 155 | 2122 ± 48 | 2.46 |
| | 110.1 | 197 | 1758 ± 38 | 3.24 |
| | 138.7 | 202 | 3012 ± 78 | 2.32 |
| | 207.7 | 134 | 6706 ± 172 | 2.36 |
| | 106.3 ± 22.5[d] | 145.7 ± 18.1 | 3269 ± 710 | 2.42 ± 0.20 |
| FG | 41.6 | 53 | 3765 ± 138 | 2.12 |
| | 47.3 | 72 | 3266 ± 128 | 2.05 |
| | 55.2 | 114 | 4622 ± 82 | 1.17 |
| | 67.0 | 144 | 3309 ± 54 | 1.44 |
| | 95.9 | 154 | 3288 ± 64 | 1.93 |
| | 145.9 | 137 | 5018 ± 98 | 2.16 |
| | 146.0 | 118 | 3907 ± 65 | 3.23 |
| | 154.5 | 131 | 5363 ± 85 | 2.24 |
| | 187.1 | 132 | 3646 ± 81 | 3.96 |
| | 217.2 | 167 | 5160 ± 91 | 2.57 |
| | 115.8 ± 18.8 | 122.2 ± 10.6 | 4134 ± 248 | 2.29 ± 0.24 |
| ALL | 97.0 ± 13.7 | 121.2 ± 9.4 | 3384 ± 331 | 2.35 ± 0.14 |

[a] in mN
[b] in $\mu m^2$
[c] in $kg/cm^2$
[d] mean ± standard error

Table 2.1 SUMMARY OF THE GLYCOGEN DEPLETED MOTOR UNIT DATA

B. REINNERVATED

| | FORCE[a] | FIBRE NUMBER | MEAN AREA[b] | SPECIFIC TENSION[c] |
|---|---|---|---|---|
| SO | 30.1 | 70 | 2415 ± 31 | 1.82 |
| | 73.8 | 153 | 2582 ± 111 | 1.91 |
| | 47.4 | 70 | 2318 ± 57 | 2.97 |
| | 48.5 | 80 | 1694 ± 81 | 3.64 |
| | 49.9 ± 7.8[d] | 93.3 ± 17.4 | 2252 ± 168 | 2.59 ± 0.38 |
| FOG | 24.5 | 63 | 2203 ± 91 | 1.79 |
| | 29.0 | 73 | 2641 ± 91 | 1.53 |
| | 36.8 | 74 | 2028 ± 43 | 2.50 |
| | 51.4 | 100 | 1859 ± 40 | 2.82 |
| | 112.2 | 208 | 2293 ± 37 | 2.40 |
| | 126.7 | 214 | 2263 ± 37 | 2.67 |
| | 174.0 | 177 | 3683 ± 54 | 2.72 |
| | 79.2 ± 20.4 | 129.9 ± 23.5 | 2424 ± 212 | 2.36 ± 0.17 |
| FInt | 35.7 | 101 | 1964 ± 61 | 1.84 |
| | 75.2 | 120 | 3749 ± 74 | 1.70 |
| | 86.0 | 128 | 1962 ± 77 | 3.49 |
| | 116.9 | 221 | 3031 ± 60 | 1.78 |
| | 158.8 | 245 | 1986 ± 42 | 3.33 |
| | 278.1 | 374 | 2534 ± 57 | 2.99 |
| | 125.1 ± 31.9 | 198.2 ± 38.8 | 2538 ± 273 | 2.52 ± 0.31 |
| FG | 114.1 | 150 | 3707 ± 89 | 2.09 |
| | 198.3 | 228 | 2558 ± 52 | 3.46 |
| | 266.3 | 213 | 5425 ± 93 | 2.35 |
| | 192.9 ± 35.9 | 197.0 ± 19.5 | 3897 ± 681 | 2.63 ± 0.34 |
| ALL | 104.2 ± 16.7 | 153.1 ± 17.7 | 2645 ± 195 | 2.49 ± 0.15 |

[a] in mN
[b] in $\mu m^2$
[c] in $kg/cm^2$
[d] mean ± standard error

### 2.III.4(b) Muscle Unit Fibre Areas

Cross-sectional fibre areas varied over a 2- to 6-fold range within single units in normal muscle (Figure 2.10). This is consistent with the 6-fold range observed previously between all units in rat TA (Kugelberg and Edstrom, 1968) and the 2 to 8-fold range in cat TA (Bodine et al., 1987). The smallest range is seen in the SO units with a larger range in type F units. The lowest and highest extremes of the range move to progressively higher values as one moves from SO to FG. Due to this large range in fibre area within a single unit, the standard error of mean unit area is large (see Table 1A). The range in fibre areas is even greater after reinnervation, 2.5 to 15-fold, and was not type-specific as in normal units.

The mean fibre area of single units was computed and the distribution of these mean values is shown in Figure 2.11. In normal muscle, the mean fibre area for different units varies between 1135.4 ± 44.6 $\mu m^2$ and 6706.0 ± 172.2 $\mu m^2$ and between 1694.1 ± 80.8 $\mu m^2$ and 5425.3 ± 92.9 $\mu m^2$ in reinnervated muscles. After reinnervation the distribution becomes skewed to the right with an overall decline in the mean fibre area (distribution mean 2644.7 ± 195.1 compared to the normal value of 3384.7 ± 330.5 $\mu m^2$). This change is consistent with the reduced ordering of unit fibre area from S = FR < FI < FF seen in Table 2.1. Mean muscle unit fibre area correlated well with force in normal muscles but less well in reinnervated muscles (Figure 2.11 C and D). The relationships are significant in both muscles (m= 0.49 ± 0.10 in normal and 0.19

Figure 2.10. The range in muscle fibre areas within normal and reinnervated motor units. Muscle fibre areas measured from each glycogen depleted motor unit (in $\mu m^2$) are expressed as a range from smallest to largest. The normal units in A show an increased range with size from type SO to FG while the reinnervated units in B show more similar size ranges in all unit types.

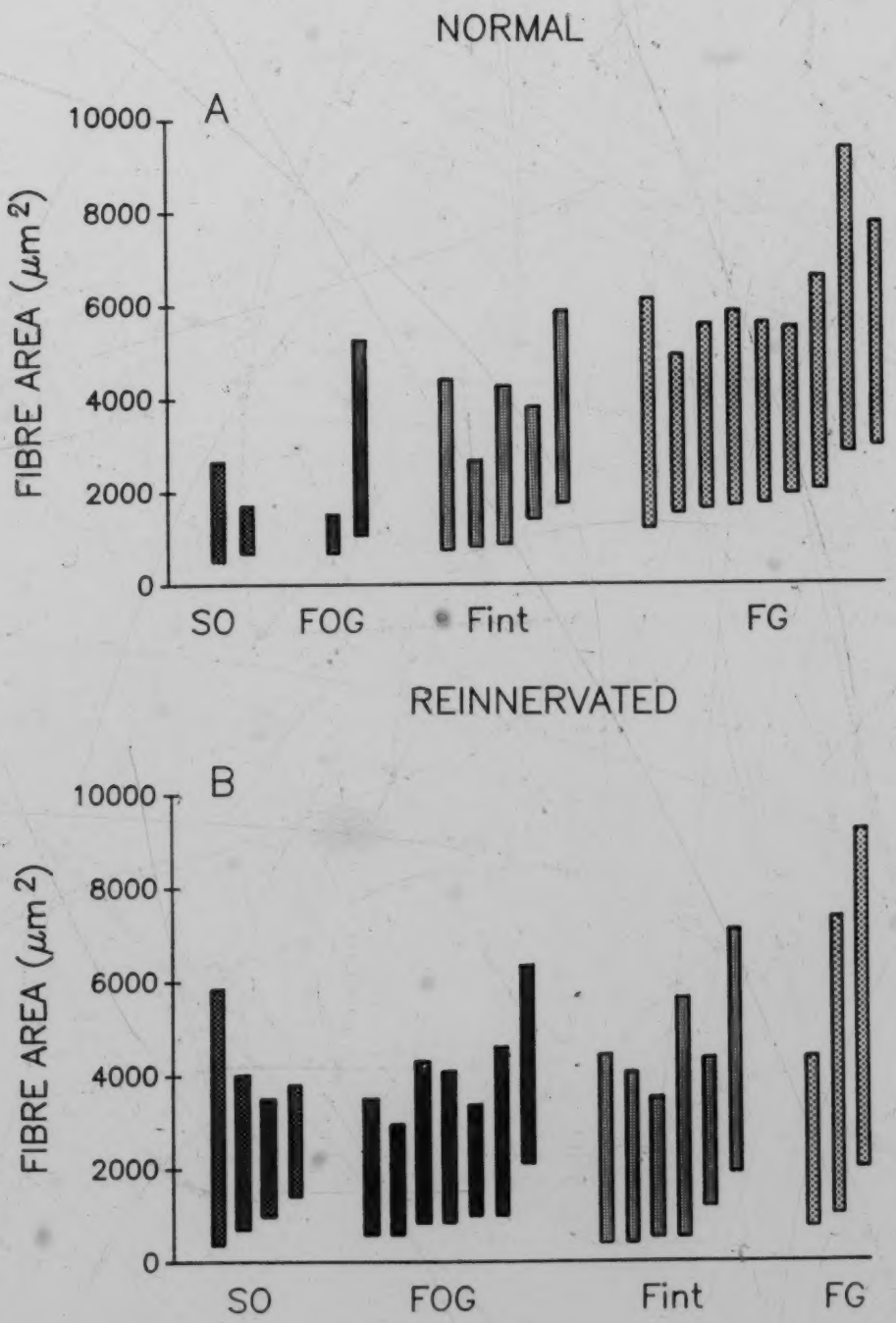
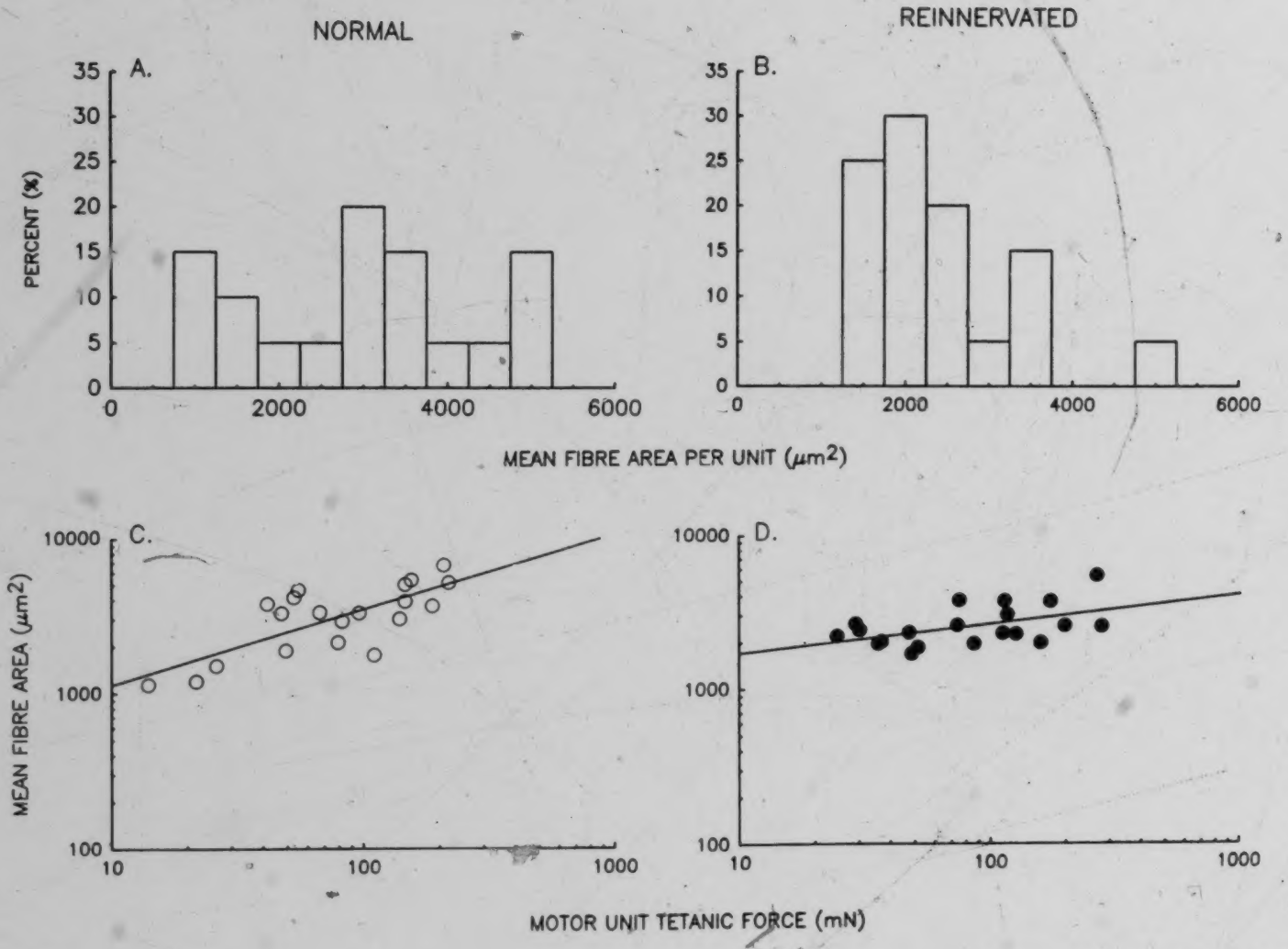

NORMAL
A
10000
8000
6000
4000
2000
0
FIBRE AREA (μm²)
SO
FOG
Fint
FG
REINNERVATED
B
10000
8000
6000
4000
2000
0
FIBRE AREA (μm²)
SO
FOG
Fint
FG

Figure 2.11. Relation between motor unit tetanic force and mean muscle fibre area per unit. The cross-sectional areas of all fibres were measured (in $\mu m^2$) for each unit and a mean value calculated. A and B show the distributions of mean fibre area and C and D shows the relation of mean fibre area to tetanic force. The regression lines have slopes of 0.49 ± 0.10 ($r=0.74$) for normal and 0.19 ± 0.08 ($r=0.60$) for reinnervated units.

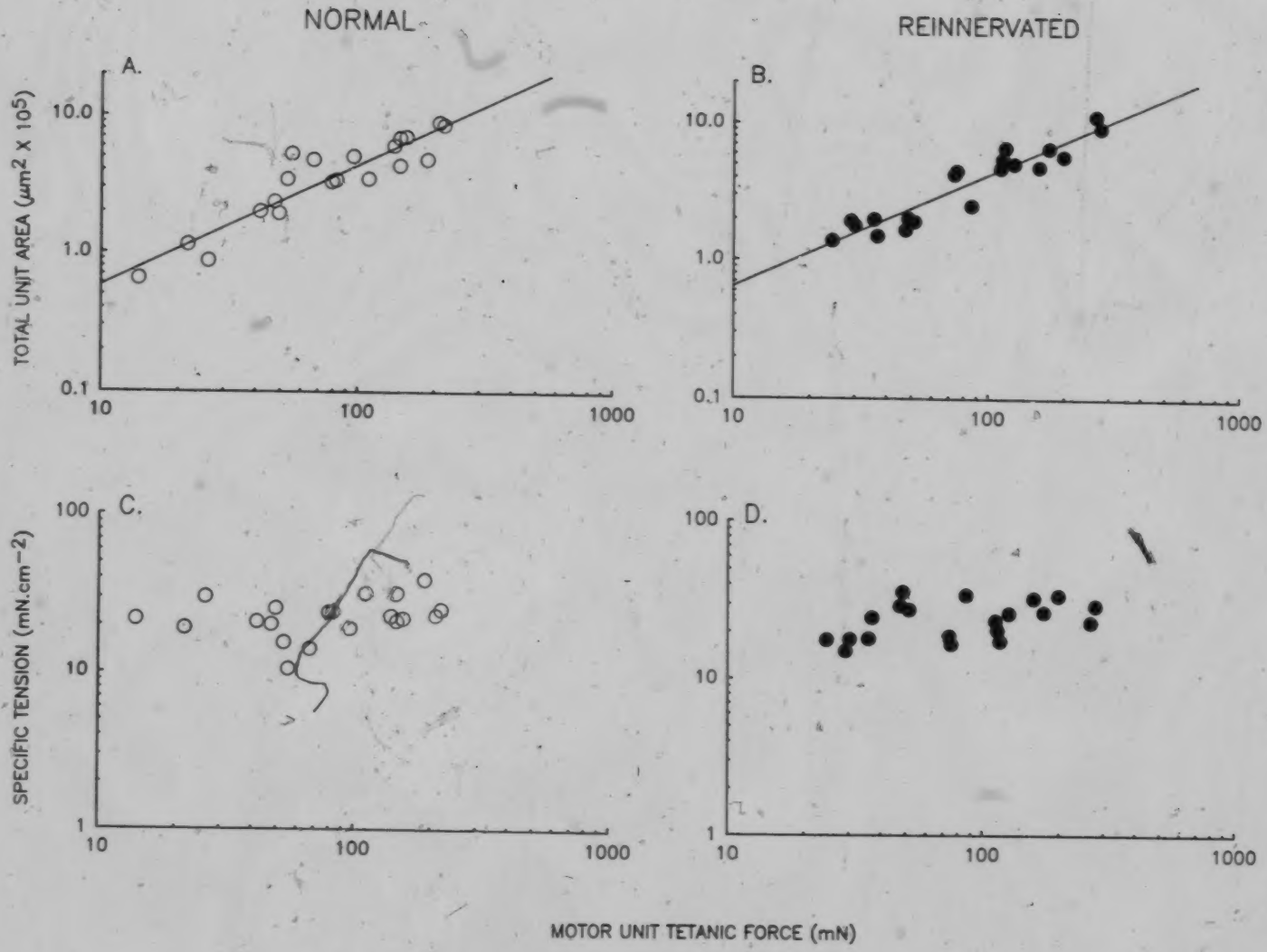

NORMAL
REINNERVATED
A.
B.
PERCENT (%)
35
30
25
20
15
10
5
0
0
2000
4000
6000
MEAN FIBRE AREA PER UNIT (μm2)
C.
D.
MEAN FIBRE AREA (μm2)
10000
1000
100
10
100
1000
MOTOR UNIT TETANIC FORCE (mN)

± 0.08 in reinnervated) and differences in force output are accounted for by mean fibre area to the extent of 49% in normal and 19% in reinnervated units. Thus fibre area is a major contributing factor to force development in normal units but contributes much less in reinnervated units.

### 2.III.4(c) Specific Tension

Although the relative contributions of fibre number and fibre area with respect to force output are very different in normal and reinnervated units, the two factors together (total unit area) correlate very well with force (Figure 2.12 A and B). Total unit area accounts for nearly all of the observed variation in force: m=0.88 ± 0.09 (r=0.92) in normal units and 0.84 ± 0.08 (r=0.93) in reinnervated units. No correlation was found between specific tension and force (C and D) with the slope not significantly different from zero. Values for specific tension, estimated by dividing maximum unit tetanic force by total cross-sectional area, ranged from 1.1 to 3.9 $kg/cm^2$ in normal (mean ± SE = 2.35 ± 0.14) and from 1.53 to 3.64 $kg/cm^2$ in reinnervated units (mean ± SE = 2.49 ± 0.15). These values were not significantly different between types (see Table 2.1) which is in agreement with the direct measurements on single skinned cat MG and SOL fibres by Lucas et al. (1987) but in contrast to the significant difference between slow and fast motor unit types found in the cat TA (Bodine et al., 1987).

Figure 2.12. Relation between motor unit tetanic force, total unit cross-sectional area and specific tension. A and B show that total unit cross-sectional area increases with force in both normal and reinnervated motor units. The slope ± standard error in A is 0.88 ± 0.09 (r=0.92) and in B is 0.84 ± 0.08 (r=0.93). C and D show that specific tension remains essentially constant as force increases.


NORMAL
REINNERVATED
A.
B.
C.
D.
TOTAL UNIT AREA (μm² X 10⁵)
SPECIFIC TENSION (mN.cm⁻²)
MOTOR UNIT TETANIC FORCE (mN)
10.0
1.0
0.1
100
10
1
1000

## 2.IV. DISCUSSION

There has been considerable controversy over whether the observed range of motor unit forces within a given muscle is due mainly to size related differences in motoneurone branching, as predicted by the size principle, or whether differences in muscle fibre size and/or force generating capacity are more important (Burke and Tsairis, 1973; Dum et al., 1982; Gordon et al, 1988). The return of the size principle after reinnervation (Gordon and Stein, 1982b) implies that important organizing principles established during development are restored. In this study we have used direct methods to test how well motor unit size correlates with 1) the extent of motoneurone branching (innervation ratio), 2) mean muscle fibre cross-sectional area, and 3) intrinsic force capacity (specific tension) in both normal and reinnervated muscles. The major findings are that the number of muscle fibres is a significant determinant of force in both normal and reinnervated units but muscle fibre cross-sectional area becomes less well correlated with force after reinnervation. This implies that the reinnervating motoneurone restores the normal range of force by controlling the number of its branches and that this compensates for the poor control of muscle fibre size. Specific tension did not vary significantly with force in either normal or reinnervated units. Before considering these results, the underlying assumptions and technical aspects associated with this investigation merit

discussion.

### 2.IV.1 Glycogen Depletion and Unit Isolation

Recently, Barker et al. (1977) found incomplete depletion of glycogen along the length of intrafusal and fatigue resistant extrafusal fibres in cat peroneus brevis muscle in response to fatiguing stimulation via $\beta$-skeletofusimotor axons as well as $\alpha$-axons. Additionally, both Edstrom and Kugelberg (1968) and Burke and Tsairis (1973) have reported difficulties in depleting slow fatigue resistant motor units. Nemeth et al. (1986) have reported occasional aberrant depletions of a small number of fibres of a different histochemical type; an observation also made by Edstrom and Kugelberg (1968). These reports have led to some doubt (see review by Burke, 1981) whether the glycogen depletion technique can provide accurate determination of muscle fibre number (innervation ratios) in all unit types. In the present study, examination of cross-sections taken from different proximodistal levels over the middle third of the muscle gave no indication of incomplete glycogen depletion along this length of fibres. A similar completeness of glycogen depletion was reported for the cat TA (Bodine et al., 1987a) and these authors have suggested that the protocol used by Barker et al. (1977) - stimulation at 30 Hz with alternating occluded and normal blood supply each for 1 minute continued for a total of 32 minutes - was insufficient to

deplete FR and S units. Similarly, Edstrom and Kugelberg (1968) used 10 Hz stimulation for 10 minutes. Burke and Tsairis (1973) and Nemeth et al. (1986) used a continuation of the fatigue test (40 Hz) for a maximum of 60 minutes. Considering that the fatigue resistant units in both Bodine's and the present study received 2 hours or more of continuous stimulation, it seems quite likely that in these previous investigations the stimulation was insufficient.

In this study, we have also observed the occasional aberrant depleted fibre of dissimilar histochemical type and since they represented a small fraction of the total (only 2 or 3 fibres out of 100 - 200), these fibres were excluded as spurious depletions. Indeed, Brandstater and Lambert (1973) reported no exceptions in the histochemical identity of depleted fibres within the units they studied. In a more recent study Nemeth et al. (1988) report that only 2 or 3 out of about 200 depleted fibres examined from 10 motor units were of a dissimilar type.

Muscles having a complex and highly angled architecture require a complete reconstruction of the muscle unit to satisfactorily determine muscle fibre counts (Burke and Tsiaris, 1973). This is not the case in the TA muscle for the following reasons: Hagbarth and Wohlfart (1952) have reported that a large number of muscle fibres from the cat TA extend through the entire length of the muscle. Some were shorter than the muscle either because they merged into the intramuscular part of the tendon or they originated from the tibia. It was later

estimated that (although primarily from the more superficial regions) muscle fibres in the cat TA extend at least 60% of the total muscle length, and have a nearly parallel orientation relative to the line of force (Powell et al., 1984). Examination of serial longitudinal sections from rat TA in this study provided no clear evidence for short fibre lengths thus we have assumed that the maximum fibre counts represent good estimates of the total number of muscle fibres belonging to each unit.

An alternative approach to identify activated muscle unit fibres involves the use of 2-deoxyglucose (2-DG) (Toop et al., 1982; Nemeth et al., 1988). This compound competes with glucose for entry into muscle fibres but is resistant to metabolism once phosphorylated and accumulates inside the cell (Sokoloff et al., 1977). Toop et al. (1982) measured the uptake of [$^{14}$C]2DG in 2 FF and 3 FR muscle units from cat FDL and found an increased density of silver grains on the autoradiograms in the same muscle fibres that were depleted of glycogen. A similar correspondance between glycogen depleted FF fibres and uptake of 2-DG has been shown with quantitative biochemical techniques (Nemeth et al., 1988). In contrast, in 2 slow muscle units from cat soleus increased autoradiographic labelling occurred in the absence of glycogen depletion (Toop et al.,1982). It can be argued that since energy production in slow twitch muscle relies primarily on blood glucose, one would expect that these slow fibres would take up detectable amounts of 2-DG before glycogen is

completely depleted. Furthermore, slow twitch units require less ATP to generate a given force than fast twitch units (Kushmerick, 1985). In summary, the results from the 2-DG studies discussed above, corroborate the results from glycogen depletion in fast twitch muscle units but not in slow units. These slow units were not stimulated for as long as in the present study and ischemia was not used to enhance glycogen breakdown. Ultimately, whatever method is used, the ability to detect slow muscle unit fibres depends on how they are activated.

Thus, if motor unit stimulation is sufficient, the identification of fibres depleted of glycogen is unambiguous. Typing the glycogen depleted fibres according to ATPase and NADH histochemistry provides a further check that they belong to the same motor unit. In newly reinnervated muscles, fibres in the same unit express both fast and slow properties but undergo transitions to become uniform histochemically. The present studies were carried out at a time when this transition was complete to avoid any ambiguity.

### 2.IV.2 Determinants of Force in Normal Muscle

The results from the present study as well as that of Bodine et al. (1987) provide direct evidence in support of Eccles and Sherrington's (1930) prediction that large $\alpha$-motoneurones branch more extensively than small ones. The number of fibres is a significant determinant of force in normal muscles and

accounts for 39% of the force in rat TA and 64% in cat TA (Bodine et al., 1987a). Differences in fibre area account for 49% and 18% so that together, fibre number and area account for 88% and 82% of the variation in force in the two studies. This is in direct contrast to the results from a series of investigations, in which fibre number (IR) has been calculated indirectly, where specific force and not IR was the major determinant (see Tables 2.2 and 2.4). The basis for the discrepancies between direct and indirect studies is considered in detail in Chapter 3.

The reason for the different contribution of fibre number and area found by Bodine et al. (1987) in cat TA and the present study on rat TA seems to be largely due to a different degree of variability in mean unit cross-sectional areas. Some differences may be expected because of the larger sample size in this study. While maximum tetanic force of these cat muscle units varied over more than a 10-fold range, mean unit fibre areas ranged from 2149 to 3694 $\mu$m2. Thus the largest units contained fibres only 1.5 times bigger on average than the smallest units. Burke and Tsairis (1973) reported a 3-fold range in mean fibre area amongst 3 cat MG muscle units. In the present study the mean unit cross-sectional areas in normal muscle ranged from 1135 to 5363 $\mu$m2 which represents a 5-fold variation. Thus the range in mean unit area is more than 3 times larger in the rat compared to the cat TA which corresponds to the difference in weighting of this factor between the two studies.

Table 2.2. Comparison of direct and indirect measurement of innervation ratios (IR).

A. INDIRECT MEASUREMENTS

| S | | FR | | FF | | |
|---|---|---|---|---|---|---|
| IR | Tet F | IR | Tet F | IR | Tet F | MUSCLE |
| 629 | 6.0 | 524 | 27.8 | 683 | 89.7 | MG[a] |
| 534 | 5.7 | 440 | 37.4 | 758 | 67.8 | MG[b] |
| 531 | 7.0 | 582 | 13.0 | 654 | 61.0 | MG[c] |
| 194 | 1.1 | 142 | 5.3 | 357 | 30.0 | FDL[d] |
| 393 | 9.6 | 363 | 30.6 | 547 | 109.9 | TP[e] |
| 229 | 2.6 | 183 | 8.9 | 254 | 21.2 | LG[f] |

[a]Gordon et al. (1988),
[b]Burke and Tsairis (1973),
[c]Foehring et al. (1986),
[d]Dum et al. (1985),
[e]McDonagh et al. (1980),
[f]Gillespie et al. (1987).
(a-e are cat muscles, f is rat)
abbreviations: MG = medial gastrocnemius, FDL = flexor digitorum longus, TP = tibialis posterior, LG = lateral gastrocnemius; Tet F = tetanic force in grams

B. DIRECT MEASUREMENTS

| S | | FR | | FI | | FF | |
|---|---|---|---|---|---|---|---|
| IR | Force | IR | Force | IR | Force | IR | Force |
| --- | --- | 132 | 0.8* | --- | --- | 132 | 1.8*[g] |
| 93 | 4.0 | 197 | 10.3 | 311 | 28.9 | 255 | 21.2[h] |
| --- | --- | 500 | 39.0 | --- | --- | 750 | 120.0[i] |
| | | | | | | 300 | 35.0 |
| 70 | 2.1 | 102 | 6.3 | 166 | 12.3 | 119 | 12.4[j] |

*Twitch force is expressed here, all others are tetanic force in grams
[g]Edstrom and Kugelberg (1968); rat TA; mean values
[h]Bodine et al. (1987); cat TA; mean values
[i]Burke and Tsairis (1973); cat MG; values from 3 units
[j]present study; rat TA; mean values from *physiological* types

From the indirect studies summarized in Table 2.2, estimated innervation ratios are often higher for type S units compared to type FR yet force is 70-80% smaller. The difference in area between SO and FOG fibres is too small to account for this difference in force. One must conclude based on these measurements that slow fibres are considerably weaker than fast - that is, their specific tension values are very much lower - yet this is not seen from the direct measurements of specific tension (Tables 2.1 and 2.3).

### 2.IV.3 Determinants of Force in Reinnervated Muscle

In reinnervated units the number of muscle fibres innervated is also a significant determinant of force and accounts for more of the observed variations (65%) between units than in normal muscle. Differences in mean fibre area account for 19% of the force. Within units there is a broader range of fibre areas with many SO fibres larger than normal and many FG fibres smaller than normal (see Figure 2.10) with the result that the mean unit fibre areas become more similar. Thus, the reinnervating motoneurone appears incapable of adjusting the size of its constituent fibres. Despite these differences from normal muscle, fibre number and area still account for most (84%) of the variations in force.

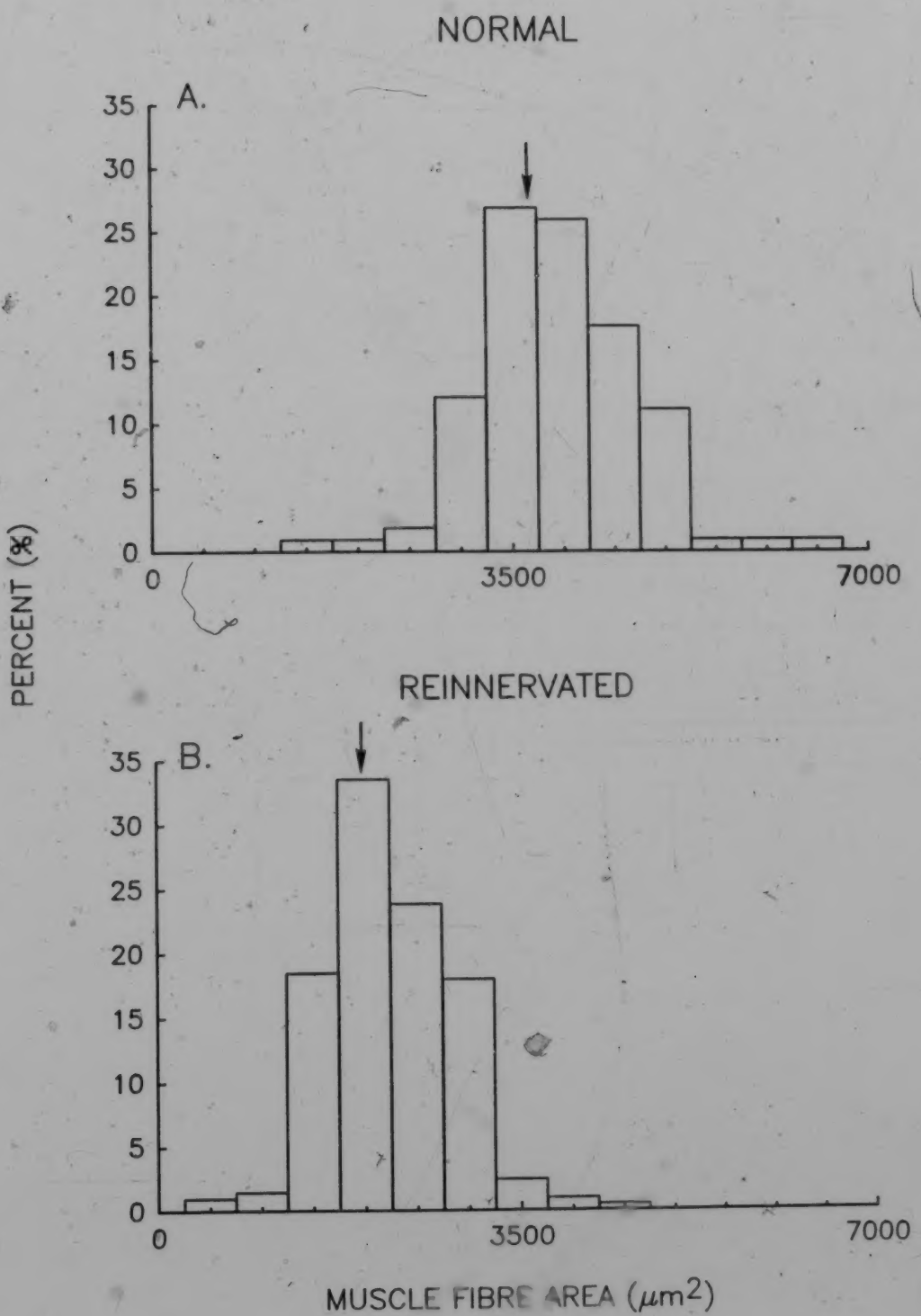
The differences in force between FF and S units that exist in normal muscle partly because of the large differences in mean muscle fibre size are produced

in reinnervated units by a greater difference in muscle fibre number. Two fast units which provide an example of this are shown in Figure 2.13. Both units developed similar force (187 and 159 mN for normal and reinnervated, respectively) but had markedly different fibre counts (132 for normal and 245 for reinnervated). It is clear from the histogram that large differences in fibre area account for this difference in fibre number. This is true for the whole population of depleted units since mean fibre number is 21% higher and mean fibre area is 22% smaller in reinnervated units compared to control (see Table 2.1). It should be noted that, although the force distributions of the normal and reinnervated depleted motor units are similar (Figure 2.6), the proportion of unit types is different. Additionally, since the whole common peroneal nerve was resutured, the composition of the motoneurone pool supplying the TA is likely different from normal and will vary between animals.

### 2.IV.3 Specific Tension

Estimates of specific tension, obtained for each unit by dividing maximum tetanic force by total cross-sectional area, showed no systematic variation between units of different size or type and contributed little to the variation in force between units in both normal and reinnervated muscle. These results compare well with direct studies using single skinned fibres of different types

Figure 2.13. Variation in muscle fibre size within a single motor unit from normal and reinnervated muscle. The normal unit in A had fibre sizes ranging from 1238 to 6143 $\mu m^2$ (5-fold) with a mean ± standard error of 3646 ± 80 $\mu m^2$ and the reinnervated unit in B had fibre sizes ranging from 498 to 4073 $\mu m^2$ (8-fold) with a mean value of 1986 ± 42 $\mu m^2$.


NORMAL
A.
35
30
25
20
15
10
5
0
0
3500
7000
PERCENT (%)
REINNERVATED
B.
35
30
25
20
15
10
5
0
0
3500
7000
MUSCLE FIBRE AREA ($\mu m^2$)

from the same muscle (Lucas et al., 1987; Laszewski, 1987; see Table 2.3). The results from the motor unit study by Bodine et al. (1987) showed a small but significantly higher specific tension in muscle fibres of fast compared to slow motor units. A higher value was also found for fast fibres using isolated intact fibres from 'fast' and 'slow' muscles (Table 2.3). Since the isolated skinned fibre tensions were measured at maximal activation (pCa of 3.6) and optimal sarcomere length, it is conceivable that the specific tensions of fast and slow fibres may be different in vivo. As discussed by Close (1972), if fast fibres do have a measurably higher specific tension in vivo, it is probably associated with differences in activation rather than differences in intrinsic strength of the contractile material. Regardless of whether specific tensions are slightly higher in fast motor units or not, it is clear from all but the indirect studies that it plays a minor role in determining the variability in force output of units of different size or type.

Considering the indirect studies, where averaged values from physiological and histochemical sampling are compared, specific tension values for slow motor units are consistently about half that of the fast units (Table 2.4) while differences in innervation ratios between types are smaller than observed from direct measurements (Table 2.2). The methodology applied in the indirect studies requires that the physiologically typed motor units directly correspond to the histochemically typed muscle fibres. Suppose, for example, more fibres exhibit

Table 2.3 DIRECT MEASUREMENTS OF SPECIFIC TENSION.

A. Whole muscle or fibre bundles

| FAST | | SLOW* | SPECIES |
|---|---|---|---|
| 2.9-3.0 | EDL | 1.9-2.1 | rat[a] |
| 1.8 | MG | 2.28 | rat[b] |
| | | 2.15 | mouse[c] |
| 3.8 | FDL | 2.50 | cat[d] |
| 3.9 | FDL | 2.90 | cat[e] |
| 1.6 | PSOAS | 0.90 | rabbit[f] |

*soleus

B. Type identified single fibres

| TYPE II | | TYPE I | | SPECIES |
|---|---|---|---|---|
| 2.43 | MG | 2.45 | MG | cat[g] |
| | | 2.30 | SOL | |
| 2.0 | EDL (IIA) | 1.5 | SOL | |
| 2.4 | EDL (IIB) | 1.8 | AL | rat[h] |
| 1.5 | PG (IIA) | 2.1 | PG | |
| 2.0 | PG (IIB) | | | rat[h] |

specific tension is reported in $kg/cm^2$
abbreviations: EDL = extensor digitorum longus, MG = medial gastrocnemius, SOL = soleus, FDL = flexor digitorum longus, PG = posterior gracilis, AL = adductor longus.

[a]Barany and Close (1969)
[b]Sexton and Gerston (1967)
[c]Rowe (1969)
[d]Kean et al. (1974)
[e]Edjtehadi and Lewis (1979)
[f]Julian et al. (1979)
[g]Lucas et al. (1987)
[h]Laszewski (1987)

Table 2.4 COMPARISON OF DIRECT AND INDIRECT MEASUREMENTS OF SPECIFIC TENSION BASED ON MOTOR UNIT DATA.

A. INDIRECT

| TYPE I (SO) | TYPE IIA (FOG) | TYPE IIB (FG) | MUSCLE |
|---|---|---|---|
| 0.6 | 2.9 | 1.7 | cat MG[a] |
| 0.67 | 0.98 | 2.41 | cat MG[b] |
| 0.31 | 1.37 | 2.45 | cat MG[c] |
| 0.6 | 3.5 | 2.9 | cat FDL[d] |
| 1.5 | 3.6 | 4.9 | cat TP[e] |

[a]Burke and Tsairis (1973)
[b]Foehring et al. (1986)
[c]Gordon et al. (1988)
[d]Dum et al. (1982)
[e]McDonagh et al. (1980)

B. DIRECT

| TYPE I (SO) | TYPE IIA (FOG) | TYPE IIB (FG) | MUSCLE |
|---|---|---|---|
| --- | 2.65 | 2.0<br>1.4 | cat MG[a] |
| 1.72 | 2.11 | 2.49 | cat TA[f] |
| 2.88 | ---- | ---- | cat SOL[g] |
| 2.4 | 2.21 | 2.37 | rat TA[h] |

[f]Bodine et al. (1987)
[g]Martin et al. (1988)
[h]present study
Note that here the term direct refers to the direct determination of the parameters used to calculate motor unit specific tension (IR and total cross-sectional area).

a type I or SO histochemical profile than belong to type S motor units. The innervation ratio would be overestimated leading to an overestimated total cross-sectional area and consequently to an underestimated specific tension. In this way, small errors in the estimation of proportions can become large errors in the estimation of fibre number and specific tension.

### 2.IV.4 Mechanisms of Size-dependent Branching

As discussed above, the number of muscle fibres innervated by a motoneurone plays an important role in determining force production in both normal and reinnervated motor units. Clearly, fibre number is determined by the number of axonal branches and the observation that force is correlated with nerve fibre size (McPhedran et al., 1965; Wuerker et al., 1965; Gordon and Stein, 1982b) suggests that this branching is size dependent. Branching may be controlled by some intrinsic property of the motoneurone or by competition for endplates during postnatal maturation or both. Tatton and Theriault (1988) have recently shown in the kitten that soma volumes of MG motoneurones are unimodally distributed at birth. Yet there is evidence to suggest that prior to synapse elimination, differences in motor unit size (Brown et al. 1976; Thompson, 1987) as well as motoneurone size (Skoglund, 1966) already exist. Thus it is possible that differences in branching are already present before synapse

elimination commences. Both the size of a motoneurone (Henneman et al., 1965a) and the elimination of synapses (O'Brien et al., 1978; Benoit and Changeux, 1978) are related to the level of activity suggesting that activity may affect the extent of branching as well as enhance synapse elimination. The observations that in rabbit soleus both the extent of polyinnervation at birth and the rate of synapse elimination during maturation is greater for slow than for fast muscle fibres (Callaway et al., 1989) and that in the immature rat activity levels of slow motor units are about twice that of fast motor units (Navarette and Vrbova, 1983) are consistent with this hypothesis and further suggest that activity enhances both initial synapse formation as well as elimination. Yet in the chick, complete activity blockade during fetal development has been shown to enhance branching and lead to axonal overgrowth (Dahm and Landmesser, 1988). The latter discrepancy may be due to species differences.

Although the mechanisms controlling branching during development and maturation remain unclear, we have found that the number of branches per motoneurone in normal muscle varies over a 3.5-fold range. Soma size, whether measured in terms of diameter or total surface area, varies over approximately a three-fold range (Kernell and Zwaagstra, 1981; Burke et al., 1982; Ulfhake and Kellerth, 1982) which lends further support to the size dependent branching hypothesis.

It is well documented that polyinnervation occurs during early stages of reinnervation (McArdle, 1975; Benoit and Changeux, 1978; Haimann et al., 1981; Angaut-Petit and Mallart, 1985), thus competition for endplate sites must also occur between reinnervating nerve terminals. If it is assumed that motoneurone properties are restored after reinnervation, one might predict that activity has a similar effect on synapse elimination as during development. Although axotomized axons undergo atrophic changes (Gutmann and Sanders, 1943; Hoffer et al., 1979; Gordon and Stein, 1982a), the presence of a normal range of motor unit force together with the rematching of axon size with contractile force and speed in reinnervated units (Gordon and Stein, 1982b; Gordon et al., 1986; Foehring et al., 1986; Albani et al., 1988) suggests that the appropriate range of motoneurone properties is indeed restored. However, the relatively tight groupings of muscle fibres within much smaller terriories in reinnervated units compared to the more widespread scattered distribution in mormal muscle (Edstrom and Kugelberg, 1968; Kugelberg et al., 1970) indicates that axonal branching patterns during regeneration are substantially different from that during development. It is not known to what extent multiple synapses are formed on a given muscle fibre from the same parent axon during reinnervation. The result that activity blockade reduces the size of reinnervated motor units in competition with normally active units (Ribchester and Taxt, 1983) is inconsistent with the results in normal immature muscle, however as pointed out by Callaway et al.

(1989), it is difficult to distinguish between the effects of inactivity on regeneration, synapse formation or synapse elimination.

### 2.IV.5 Control of Muscle Fibre Size

It is well known that muscle properties are readily altered by changes in innervation or activity (Buller et al., 1960; Salmons and Vrbova, 1969; see also reviews by Pette and Vrbova, 1985; Edstrom and Grimby, 1986) indicating a strong level of neural control by the motoneurone. Recent evidence suggests that expression of muscle phenotype early in development is nerve-independent both in chick (Butler et al., 1982; Miller and Stockdale, 1987) and rat (Dhoot, 1985) muscle however this is not clear in all studies (Rubinstein and Kelly, 1981; Rubinstein et al., 1985). At the level of a single motor unit muscle fibres are not homogeneous in size (this study; Bodine et al., 1987) or in metabolic enzyme activities, although the latter point is still controversial (Nemeth et al., 1986; Martin et al, 1988). Although muscle fibre size is apparently not exclusively determined by the motoneurone, the range of fibre sizes within a motor unit is less than that of the whole population of fibres of similar type.

After reinnervation a consistent finding is that size distribution of fibres of different types show more overlap (Foehring et al., 1986; Gordon and Stein, 1982b; Gillespie et al., 1987;, Gordon et al., 1988; the present study). Type S

units include larger muscle fibres and the F units include smaller fibres such that the range of sizes within each unit type becomes similar. This implies that, like motoneurone recovery from axotomy, muscle fibres tend to recover their former size. It would appear that fibre size is influenced by local factors which may include position within the muscle. This suggestion is supported by the gradation in size from small in the deep regions to large in the superficial regions (Pullen, 1977) which appears to return after reinnervation (see Chapter 5). The importance of position is further emphasized by the observation that the distribution pattern of the fast and slow myotubes in the rat TA is determined by their position in the limb well before the innervating neurones are fully differentiated (Dhoot, 1985).

### II.IV.6. Functional Significance

Whatever the mechanisms are that control nerve branching during development, it is apparent that soma size and muscle unit fibre number vary over a similar range in the adult. We are suggesting that motoneurone size or properties associated with size determine the final number of muscle fibres a motoneurone can supply and together with muscle fibre area adjustments, ultimately determines the force capacity of the motor unit. In reinnervated muscle, in spite of the changes resulting from axotomy, the different environment through

which regenerating neurones must grow compared to that during development and the diverse population of muscle fibres innervated by the same motoneurone, branching still appears to be size dependent and can compensate for the reduced control of muscle fibre size. The functional result of this, is that the stereotyped recruitment order of motoneurones from small to large results in a recruitment order of force by determining 1) the extent of branching and 2) the size range of muscle unit fibres. The ability of the reinnervating motoneurone to compensate for reduced control over muscle fibre size by increasing the extent of branching is a new example of the striking plasticity of the neuromuscular system.

# CHAPTER 3

# COMPARISON OF PHYSIOLOGICAL AND HISTOCHEMICAL PROPERTIES

## 3.I. INTRODUCTION

Motor units in a variety of cat limb muscles cluster into groups when certain mechanical properties, such as force output, contraction speed and resistance to fatigue during prolonged stimulation, are considered together (Burke et al., 1973; Goslow et al., 1977; Dum et al., 1982; Gordon and Stein, 1982b). Electrical properties of $\alpha$-motoneurones also tend to form groups when input resistance, rheobase and conduction velocity are considered (Stevens and Stuart, 1975; Fleshman et al., 1981; Gustafsson and Pinter, 1985; Zengel et al., 1985). Units have been separated into 'fast' and 'slow' types on the basis of contraction time (Burke, 1967; Botterman et al., 1975; Fleshman et al., 1981; Gordon et al., 1988) or on the presence or absence of a decline in force ('sag') during an unfused tetanus (Burke et al., 1973). The fast population has been further subdivided into three types according to fatigue index as described by Burke and his colleagues (1973): fast, fatigue resistant (FR, with fatigue index > 0.75), fast, fatigue sensitive (FF, with fatigue index < 0.25) and fast, fatigue intermediate (FI) which fall between the two. Also, mean force output of these four unit types is ordered

such that FF > FI > FR > S.

There is considerable evidence that motor units are continuously graded with respect to electrical and mechanical properties and represent a functional continuum within which they are recruited according to cell size (Henneman et al., 1965a; Clamann and Henneman, 1976; Luscher et al., 1983; Binder et al., 1983; Stein and Dengler, 1988). Significant correlations between motoneurone size and numerous electrical properties (Kernell, 1966; Burke and Ten Bruggencate, 1971; reviewed by Stuart and Enoka, 1983) as well as between axon size (extracellular axon potential amplitude) and muscle unit contractile force and speed (Gordon and Stein, 1982b) have been reported. Additionally, the normal correlations between motoneurone electrical properties and muscle unit contractile properties return following reinnervation (Gordon and Stein, 1982b; Foehring, et al., 1986).

Histochemical staining of muscle cross-sections has been developed to classify different types of muscle fibres on the basis of metabolic enzyme activities or myosin ATPase properties (Stein and Padykula, 1962; Guth and Samaha, 1970; Brooke and Kaiser, 1970, 1974; Peter et al., 1972; Dubowitz and Brooke, 1973). With most of the available techniques at least three different staining intensities have been observed, from which tripartite classification schemes emerged. A number of different schemes using different nomenclature have been used however the nomenclatures most commonly used today are

from Brooke and Kaiser (1970) and Peter et al. (1972): Type I fibres, or slow-twitch oxidative (SO), type IIA, or fast-twitch-oxidative-glycolytic (FOG), and type IIB or fast-twitch-glycolytic (FG).

Numerous studies have reported a reasonable match between the proportions of these three different muscle fibre types and the proportions of the three major motor unit types within the whole muscle (Burke et al., 1973; McDonagh et al., 1980; Dum et al., 1982; Gillespie et al., 1987; Gordon et al., 1988). Proportions yielded by the two classification systems do not match exactly however and this has been taken to reflect differences in the average innervation ratios of each type (Dum et al., 1982). The few isolated single units that have been examined both physiologically and histochemically (Edstrom and Kugelberg, 1968; Kugelberg et al., 1970; Brandstater and Lambert, 1973; Burke and Tsairis, 1973; Dum and Kennedy, 1980a; Nemeth et al., 1986; Bodine et al., 1987; Hamm et al., 1988) have provided evidence that all muscle fibres within units are of the same histochemical type and that neuronal, contractile and biochemical properties are functionally interrelated. Reinnervated and sprouted (Kugelberg et al., 1970; Brandstater and Lambert, 1973) motor units also contain fibres of the same type.

Although motor unit physiological properties may be clustered into groups in some muscles, clustering into four distinct types is not absolute (Reinking et al., 1975; Botterman et al., 1985; Gardiner et al., 1987) and after reinnervation the

overlap of properties between types is more extensive such that clustering becomes less evident (Chan et al., 1982; Dum et al., 1985; Gordon et al., 1986; Gillespie et al., 1987; Gordon et al., 1988). Despite the overlap, a similar continuum of properties exists in both normal and reinnervated muscles. Comparison of the proportions of motor unit types and histochemical fibre types has been used to investigate the determinants of force and has led to the conclusion that specific force is the major determinant and that innervation ratio (IR) is not an important contributing factor. Implicit in Henneman's size principle, which suggests that a size dependent recruitment underlies the observed gradation of force during movement, is a size dependent branching of motoneurones. This, however, was not supported by indirect estimates of IR. We have used a muscle in which there is a continuum of properties with little distinction between types (Kugelberg and Lindegren, 1979) to compare direct and indirect methods of estimating IR's in normal and reinnervated muscle where glycogen depleted units can be classified both physiologically and histochemically. We have further examined the alteration in force and fatigue capacities of reinnervated motor units.

## 3.II. METHODS

### 3.II.1 Physiological Recording

Female Sprague Dawley rats (280 to 400 grams) were used in this study and the preparation was the same as that described in Chapter 2. Briefly, single motor units in the rat TA muscle were isolated with graded stimulation of the ventral root filament such that an all or none response was observed in both the force and EMG recordings. The following physiological characteristics of isolated units were examined: 1) peak force, the half-time and time to peak force production and half fall time during single twitches, 2) maximum peak force production during fused tetanic contractions, 3) presence or absence of a decline ('sag') from peak force during unfused tetanic contractions, and 4) relative decline in force during 2 minutes of fatiguing tetanic contractions (40 Hz tetanic trains, 1/sec). The time to peak force development obtained from 1) can be used to subdivide the population into fast-contracting and slow-contracting units and the maximum tetanic force from 2) is a measure of the size of the unit. Both 3) and 4) are tests developed by Burke et al., 1973) where 3) is designed to differentiate between fast (sag) and slow (no sag) units and 4) is designed to subdivide the fast population into fatigue resistant (fatigue index > 0.75) and

fatigue sensitive (fatigue index < 0.25) with fatigue intermediate units falling in between the two.

### 3.II.2 Glycogen Depletion

Glycogen depletion of individual motor units from muscles previously loaded with glycogen was achieved by repetitive stimulation as previously described. The blood supply was occluded to the most fatigue resistant units to enhance glycogen breakdown. A variable number of trains were delivered each second depending on the responses in both force and EMG to the stimulation. A more detailed description is provided in Chapter 2.

### 3.II.3 Histochemistry

Immediately following cessation of stimulation muscles were quickly removed, cut into blocks and frozen in isopentane cooled in liquid nitrogen. Serial sections 10 microns thick were cut from these frozen blocks and stained for 1) glycogen using periodic acid-Schiff (PAS) according to the method of Pearse (1960), 2) myosin ATPase following acid preincubation modified from Brooke and Kaiser (1970), 3) myosin ATPase following alkaline preincubation modified from Guth and Samaha (1970), and NADH according to Dubowitz and Brooke (1973).

Histochemical profiles of the three major fibre types were similar to that described previously (Peter et al., 1972; Brooke and Kaiser, 1970; and shown in Figure 3.1). Type I (or slow-twitch oxidative, SO) fibres are intensely staining on acid-ATPase, negative staining on alkaline-ATPase, and intermediate-dark on NADH. Type IIA (or fast-twitch oxidative-glycolytic, FOG) fibres are intermediate to light staining at the centre but contain a dense floccular precipitate around the periphery on acid-ATPase, and intensely staining on both alkaline-ATPase and NADH. Type IIB (or fast-twitch glycolytic, FG) fibres are light staining on all three stains. A fourth type could also be discriminated, called fast-twitch intermediate (Fint), which had intermediate intensity on all three stains. Although the profile was different, a fourth type was also reported in the cat tibialis posterior muscle (McDonagh et al., 1980).

### 3.II.4 Muscle Fibre Proportions

Four rats in each of the normal and reinnervated group were used for determination of muscle fibre proportions. Previous work of Pullen (1977) and Armstrong and Phelps (1984) has shown the rat TA muscle to be highly regionalized both with respect to muscle fibre size and the occurrence of different fibre types. For this reason, five different regions of the muscle were selected for the determination of muscle fibre proportions: deep, central, medial,

superficial and lateral. Grey level pictures generated from an image processing computer system (described in Chapter 2) were printed from serial sections stained for myosin ATPase (both acid and alkaline preincubation) and NADH from the same region of the muscle. The image processing system consists of a light microscope (Zeiss) connected to a video camera which sends a live image of the field under the miscroscope to a video display monitor as well as to a video printer. The printed pictures so obtained were small, thus each fibre in the field was typed using higher power magnification under the microscope.

### 3.II.5 Estimated Total Number of Muscle Fibres

For indirect determination of innervation ratios and specific tension the total number of fibres in the rat TA must be known. This was estimated from the product of the average muscle fibre density and the average muscle cross-sectional area. Fibre density was estimated from the grey level pictures described in the previous section. Each picture contains 512 X 512 picture elements (or pixels) but due to the constraints of the video display, pixel lengths are different in the horizontal and vertical directions. With a horizontal pixel length of 1.61 $\mu$m and a vertical length of 1.74 $\mu$m (calibrated with a stage micrometer and a 10X objective lens on the microscope) the total area represented by each grey level picture comes out to be 0.73 $mm^2$ and represents a constant field size. The

average density of fibres per $mm^2$ was obtained from counting the number of fibres contained in these constant fields from different regions of the muscle. A total of 24 fields were analyzed from 4 normal and 4 reinnervated muscles. The total cross-sectional area of each muscle was measured by planimetry from camera lucida drawings. Results of these measurements are given in Table 3.1.

## 3.III. RESULTS

### 3.III.1 Histochemical Typing

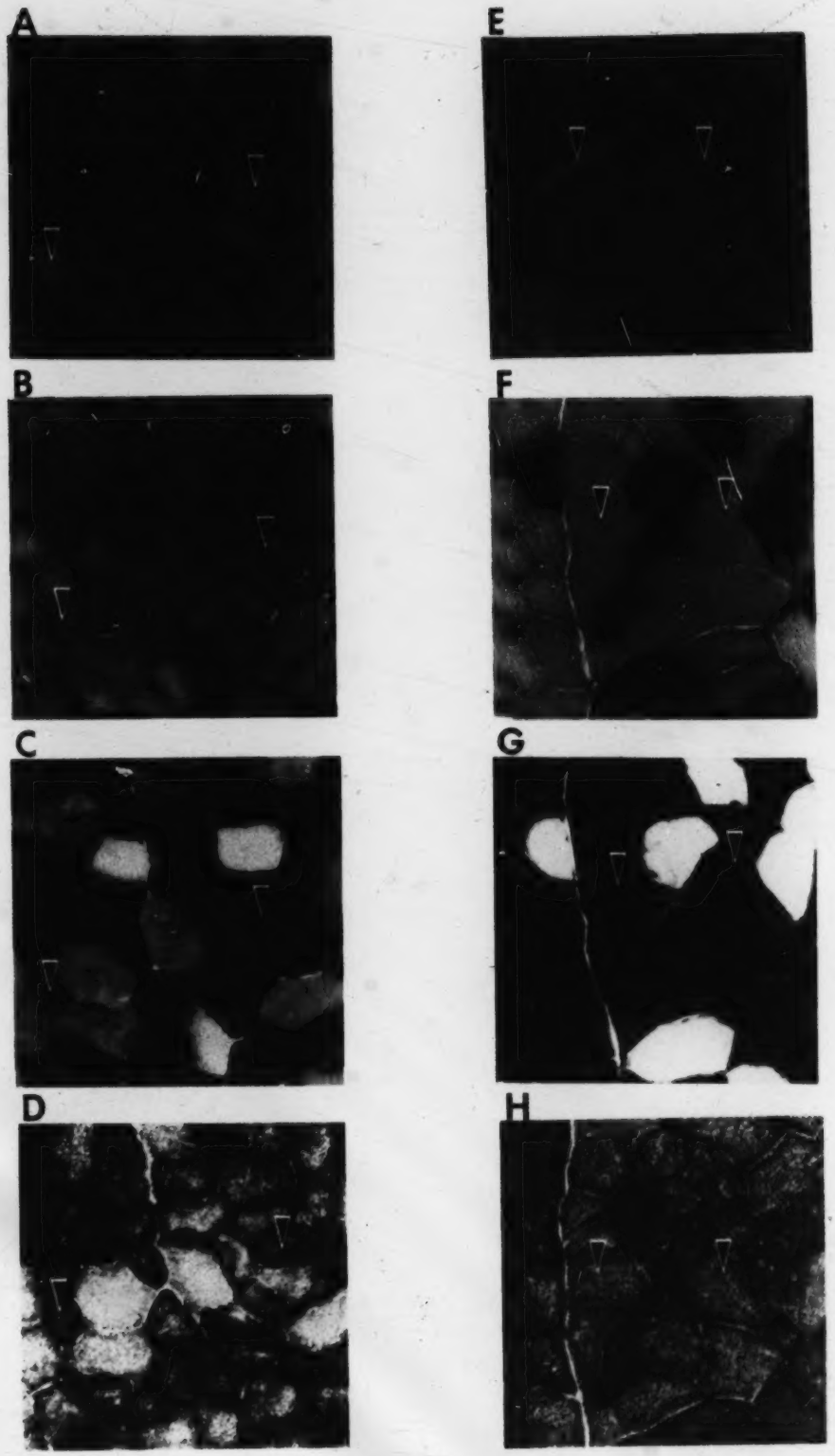
Figure 3.1 contains serial sections from a normal (A-D) and reinnervated (E-H) glycogen depleted motor unit. A and E show sections stained for glycogen (PAS), B and F for myosin ATPase with acid (pH 4.5) preincubation, C and G for myosin ATPase with alkaline (pH 10.4) preincubation and D and H for NADH. Note that muscle fibres of the stimulated motor unit are identified by their PAS-negative staining and appear white. Fibres of the normal unit are widely scattered while the reinnervated unit is clumped, confirming what has been reported previously (Edstrom and Kugelberg, 1968; Kugelberg et al., 1970). These normal depleted fibres are light staining with a darker border on the acid stain and darkest on the alkali and NADH stains, identifying the unit as FOG. The reinnervated unit fibres are pale staining with all three stains and are identified as FG.

### 3.III.2 Physiological Typing

#### 3.III.2(a) Normal Units

Analysis of the time to peak force (TTP), half rise time (1/2RT) and half fall time (1/2FT) of isometric twitch contractions in normal TA shows that their

Figure 3.1. Histochemical staining of fibres from a normal and reinnervated glycogen depleted muscle unit. A and D are PAS-stained sections (normal and reinnervated, respectively) and the fibres that appear white are part of the muscle unit. All the normal unit fibres are pale with a darker border on acid-ATPase (B), and dark on both alkaline-ATPase (C) and NADH (D) which is characteristic of type IIA or FOG. The reinnervated unit fibres are pale on acid-ATPase (F), alkaline ATPase (G) and NADH (H) which is characteristic of type IIB or FG.

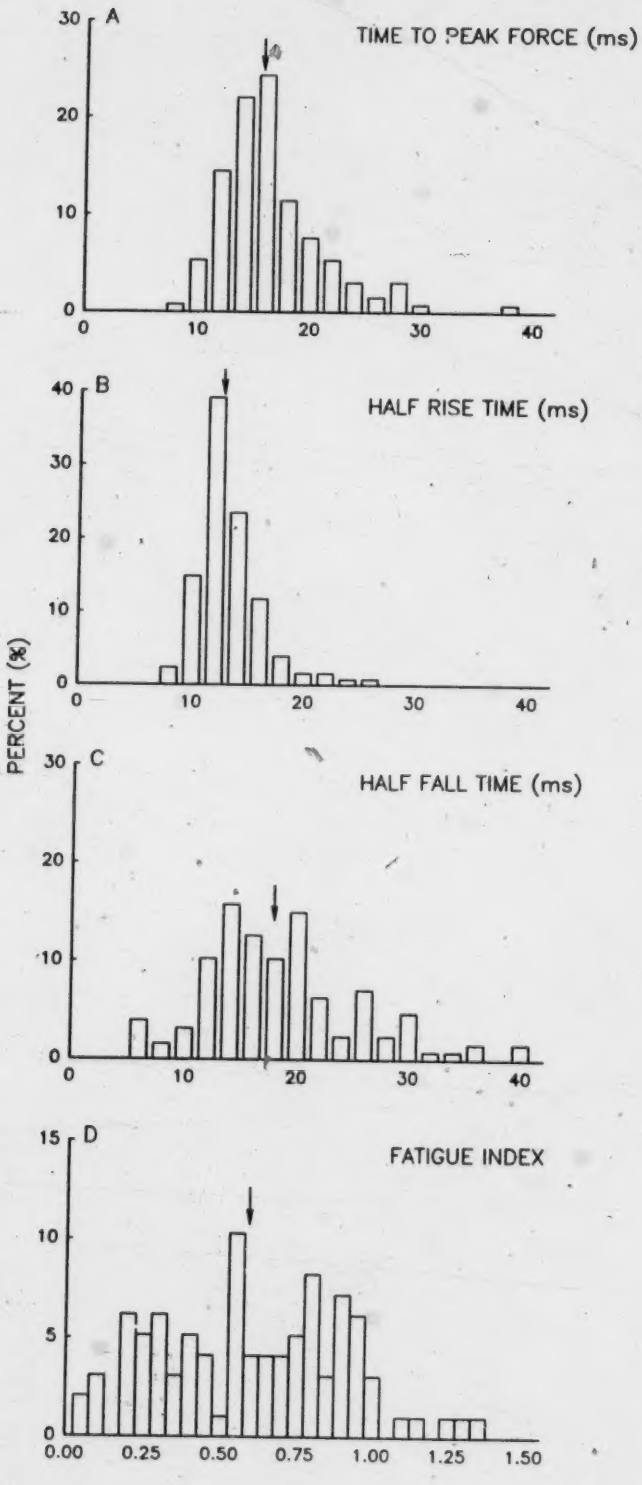

A
B
C
D
E
F
G
H

distributions are unimodal (Figure 3.2 A-C). TTP ranges continuously from 8-28 ms, with one unit at 38 ms, which is consistent with the continuous range of contraction speed seen in cat MG (Burke et al., 1973; Reinking et al., 1975), peroneus longus (Kernell et al., 1983), FDL (Dum et al., 1982), TA and EDL (Dum and Kennedy, 1980a) muscles, but inconsistent with the bimodal distributions reported by Kugleberg and Lindegren (1979) for rat TA, and by Botterman et al. (1975) for cat flexor carpi radialis (FCR). The other parameters of contraction speed also vary continuously but note that 1/2FT has a broader range (7 to 39 ms) than 1/2RT (6 to 25 ms). These distributions show no obvious breakpoint in the contraction times around which to separate fast from slow units.

Fatigability provides an independent criterion for unit typing. Using the fatigue test devised by Burke and his colleagues (1973), where the force generated by the last of 120 tetani is expressed as a fraction of the first to generate a fatigue index, units may be subdivided into fatigue resistant (fatigue index < 0.75), fatigue intermediate (fatigue index between 0.25 and 0.75) and fatigue sensitive (fatigue index < 0.25). Figure 3.2 D shows that the distribution of fatigue resistance is also continuous with just as many units at the borders between groups as at the extremes, and with a large proportion of units being fatigue intermediate. Those units showing potentiation during the fatigue test (fatigue index > 1) were all relatively fast contracting with TTPs ranging from 12 to 18 ms.

Figure 3.2. Distribution of (A) time to peak force, (B) half rise time, (C) half fall time, and (D) fatigue index in normal motor units. In each case, the distributions are unimodal providing no obvious breakpoint(s) with which to separate the population into subgroups either on the basis of speed or fatigability. The arrows denote the population mean in each case.

NORMAL


A
TIME TO PEAK FORCE (ms)
30
20
10
0
0
10
20
30
40
B
HALF RISE TIME (ms)
40
30
20
10
0
PERCENT (%)
C
HALF FALL TIME (ms)
D
FATIGUE INDEX
15
5
0.00
0.25
0.50
0.75
1.00
1.25
1.50

Either speed or sag has been used to subdivide the fast (F) and slow (S) types and fatigue index to further subdivide the fast population. In Figure 3.3 A, if all units with TTP similar to the FF types are classified as F (see Foehring et al., 1986; Gordon et al., 1988) the subdivision between F and S would be at 20 ms. However the S population then includes a number of fatigue intermediate units. The other criterion which is independent of speed is 'sag' where the unfused tetanus (at 1.25 X the contraction time) of F units declines (sags) in force over 800 ms while in S units force progressively rises (Burke et al., 1973). However sag appears to occur in 29 out of 94 F units and is therefore not a reliable criterion to separate F from S. Note that none of the S units sagged. By combining fatigue index and TTP, the fatigue resistant units can be subdivided at 22 ms as shown by the vertical line in Figure 3.3.

It should be noted that during a series of six unfused tetanic contractions the force profile changed. Nearly all normal units and more than half of the reinnervated units showed some degree of sag from peak force during the very first contraction, but little or no sag during the second and subsequent contractions. This change in shape from the first to the second contraction was accompanied by either no change or a 20-50% reduction in peak force. Force was then seen to either progressively increase back to the initial level or to remain depressed by the sixth contraction. Whichever unfused tetanic contraction was used to indicate sag, type S units never sagged but F's did or did not

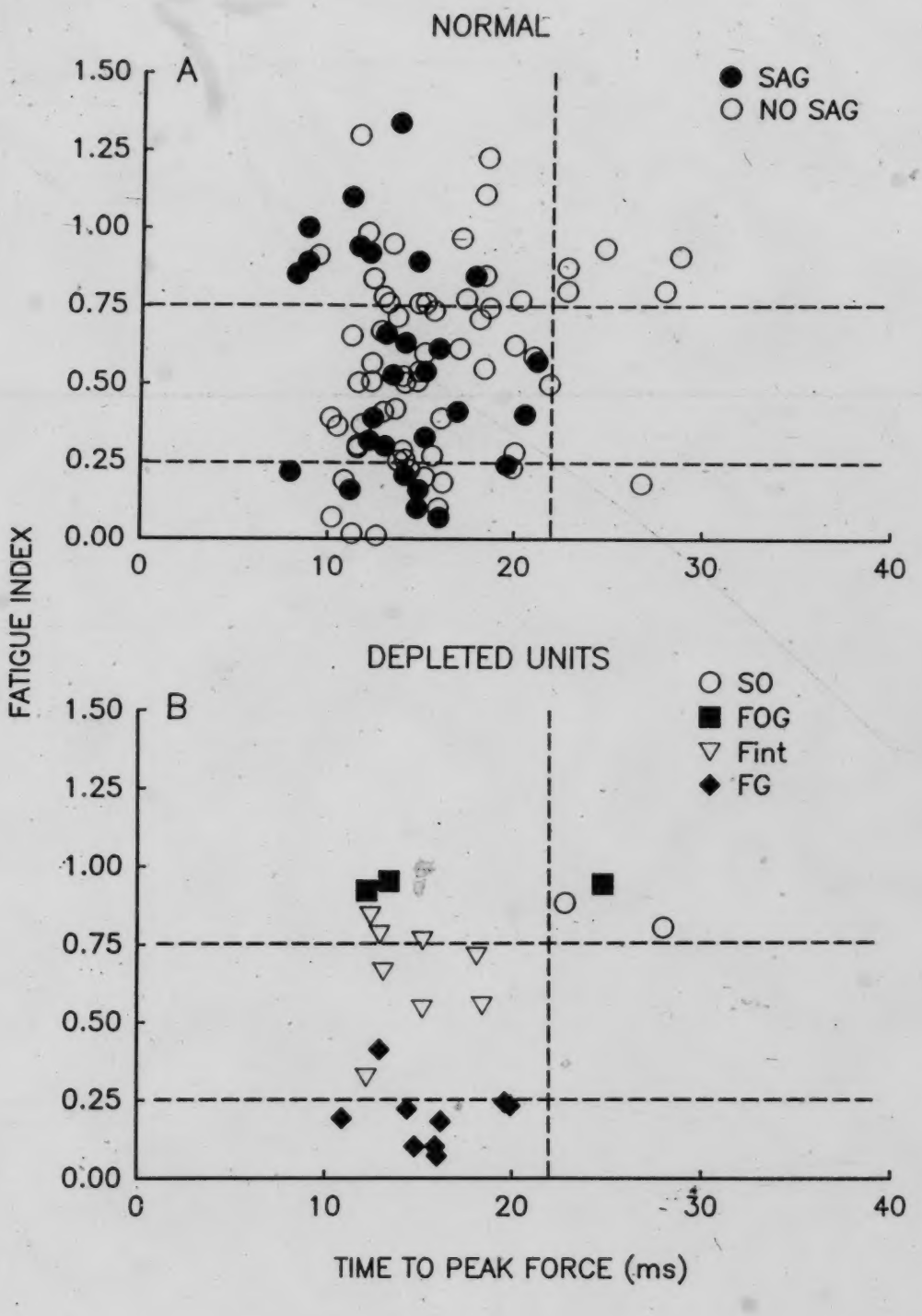
unpredictably. Additionally, the different tetanic force profiles demonstrated in motor units sampled from the 'fast' TA and 'slow' soleus muscles of the rat by Larsson and Edstrom (1987) also did not distinguish fast from slow units within the TA muscle.

If units are characterized physiologically as well as identified histochemically after glycogen depletion (Figure 3.3 B) the subdivision of F and S fails for one of three S units. Furthermore, 3 of 5 FR units are Fint. Thus the physiological classification of S and FR is subject to significant errors. All of the units classified as FF were typed FG histochemically and one FI unit was histochemically FG. It should be noted that the three discrepant FR units had a fatigue index near the border between FR and FI and thus their misclassification may be considered within the range of experimental error. The FI unit that was histochemically FG had a fatigue index of 0.41 and was thus not near the breakpoint between the two unit types. A similar discrepancy in classification has been reported for the cat TA and EDL muscles by Dum and Kennedy (1980a). Out of 3 FF and 6 FR glycogen depleted units (they do not report which units are from which muscle) two units classified as FR had histochemical profiles different from the other FR units.

### 3.III.2(b) Reinnervated Units

Contraction speed and fatigue resistance of reinnervated motor units are

Figure 3.3. Relationship between time to peak force and fatigue index in the whole sampled population and the glycogen depleted population of normal motor units. The filled symbols in A indicate the presence of sag during the unfused tetanus and the vertical broken line at 22 ms represents the experimentally determined breakpoint between fast and slow units where all units with TTP > 22 ms are considered slow. Note that all but one of these slow units are fatigue resistant. A total of 135 units were sampled from 29 rats in the whole population data. In (B) the histochemical type of the depleted muscle unit is indicated. Open circles are slow-contracting, oxidative fibres, filled squares are fast-contracting, oxidative and glycolytic fibres, open inverted triangles are fast-contracting intermediate fibres and filled diamonds are fast-contracting, glycolytic fibres. It can be seen that the physiological and histochemical typing does not always correspond. Of the 23 glycogen depleted normal motor units there were 9 FF, 6 FI, 5 FR and 3 S types. In 5 out of these 23 units the histochemical classification did not match the physiological classification.


NORMAL
A
SAG
NO SAG
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
FATIGUE INDEX
DEPLETED UNITS
B
SO
FOG
Fint
FG
TIME TO PEAK FORCE (ms)

qualitatively similar to normal in that their distributions are continuous (Figure 3.4 A-D) however there is a greater incidence of units with a TTP greater than 30 ms and a half fall time greater than 40 ms. On the whole, reinnervated motor units are more fatigue resistant with a mean fatigue index of 0.68 ± 0.03 (n=117) compared to 0.55 ± 0.03 in control (n=97). A similar finding was reported for self-reinnervated (Gordon and Stein, 1982b; Foehring et al., 1986; Cope et al., 1987) and cross-reinnervated (Gordon et al., 1988) cat hindlimb motor units where FF units became less fatigable.

In reinnervated TA there are more type S and fewer FF units present than in normal muscles, which may be related to the different population of motoneurones innervating the muscle. Cutting and resuturing the common peroneal nerve leads to regeneration back to all of the lower hindlimb flexor muscles including the TA. Cross-sections of these reinnervated muscles stained for myosin ATPase activity show a dramatic increase in the number of SO muscle fibres which suggests that more slow motoneurones go back to the TA (Totosy de Zepetnek et al., 1989).

Nearly all the reinnervated S units are fatigue resistant and do not sag, and the F units again do not consistently sag as seen in normal muscle (Figure 3.5 A). The histochemically identified units are plotted in Figure 3.5 B and it is evident that eight units have been misclassified. One out of 10 S units is Fint and 2 SO units are FR physiologically. Thus, the division of S and FR is also subject

Figure 3.4. Distribution of (A) time to peak force, (B) half rise time, (C) half fall time, and (D) fatigue index in reinnervated motor units. As in norml muscle, the distributions are unimodal providing no obvious breakpoint(s) with which to separate the population into subgroups. The arrows denote the population mean in each case.

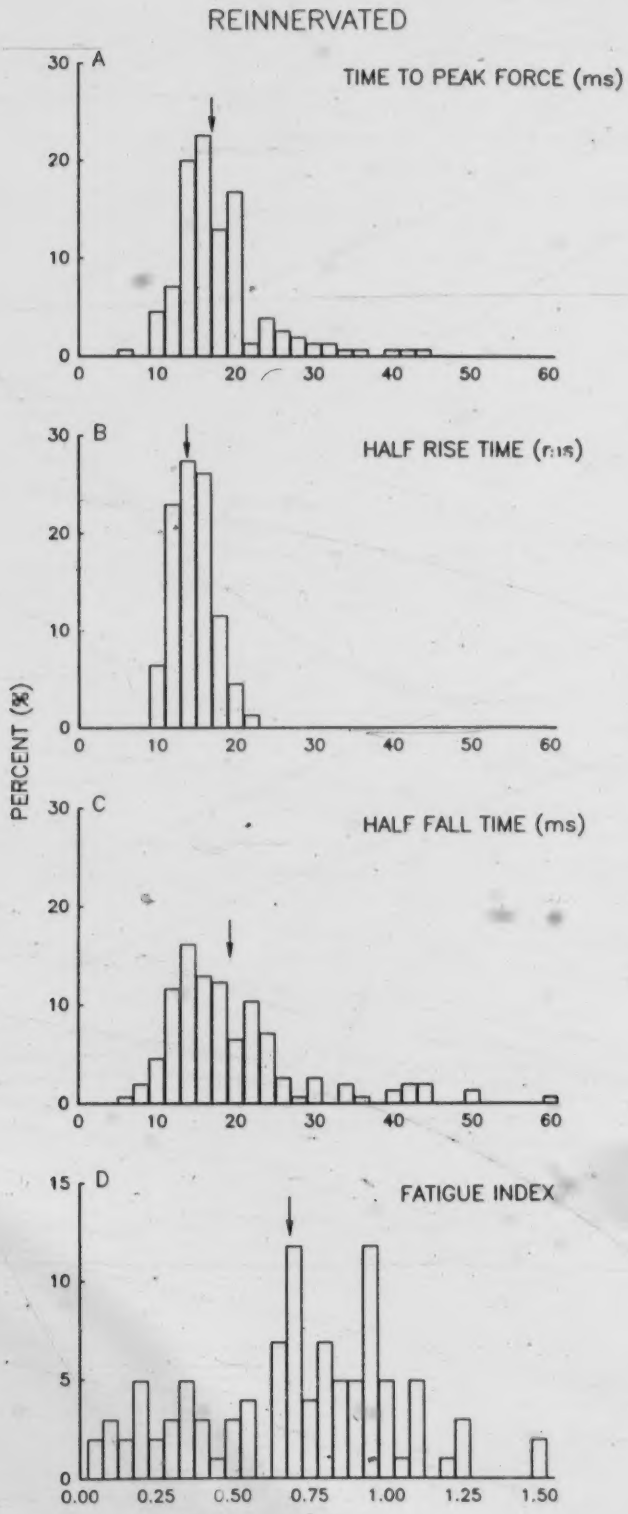
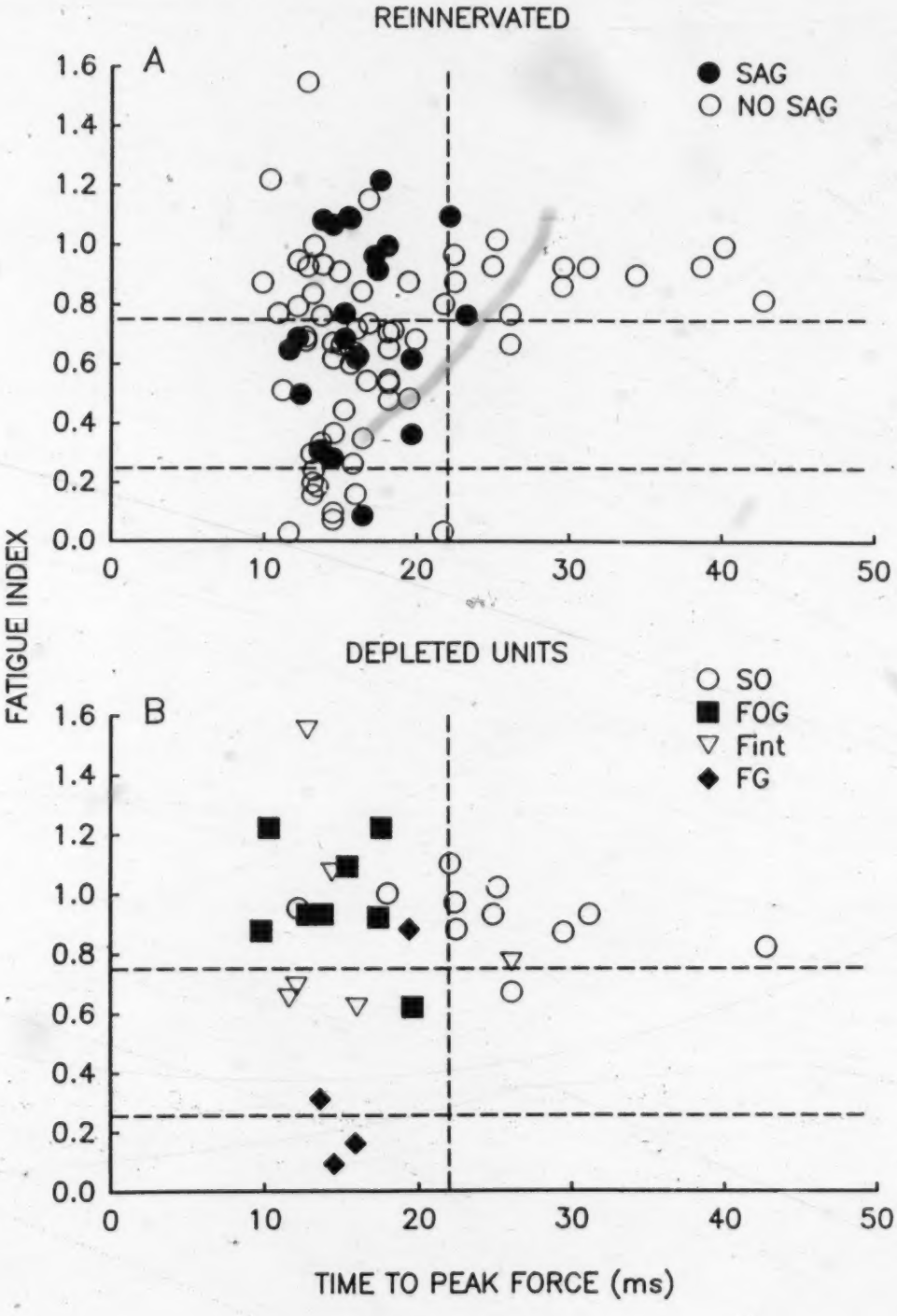

REINNERVATED
A
TIME TO PEAK FORCE (ms)
B
HALF RISE TIME (ms)
C
HALF FALL TIME (ms)
D
FATIGUE INDEX
PERCENT (%)
0
10
20
30
40
50
60
0.00
0.25
0.50
0.75
1.00
1.25
1.50
5
15

Figure 3.5. Relationship between time to peak force and fatigue index in (A) the whole sampled population and (B) the glycogen depleted population of reinnervated motor units. The filled symbols indicate the presence of sag during the unfused tetanus and the vertical broken line at 22 ms represents the experimentally determined breakpoint between fast and slow units where all units with TTP > 22 ms are considered slow. A total of 159 units were sampled from 32 rats in the whole population data. In (B) the histochemical type of the depleted muscle unit is indicated. Open circles are slow-contracting, oxidative fibres, filled squares are fast-contracting, oxidative and glycolytic fibres, open inverted triangles are fast-contracting intermediate fibres and filled diamonds are fast-contracting, glycolytic fibres. It can be seen that the physiological and histochemical typing does not always correspond. Out of 29 depleted units the histochemical types of 8 of them did not match the physiological classification. For 3 FR units the histochemical classification was unattainable and was assumed to match.


REINNERVATED
A
SAG
NO SAG
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
FATIGUE INDEX
DEPLETED UNITS
B
SO
FOG
Fint
FG
TIME TO PEAK FORCE (ms)

to significant error in reinnervated muscle. One of the eight misclassified units had a borderline fatigue index as described previously for normal muscle - an FG unit with a fatigue index of 0.31. The remaining 7 units are more distinctly mismatched thus there appears to be more substantial errors in physiological classification in reinnervated compared to normal motor units.

### 3.III.3 Motor Unit Force and Fatigability

The distribution of motor unit tetanic force in normal and reinnervated muscle is highly skewed and varies over a 100-fold range (see Chapter 2, Figure 2.3). Within this range lie the distributions of the four unit types which are given in Figure 3.6. The arrow in each case represents the sample mean. Overall, the order of increasing force is S = FR < FI < FF for normal and S < FR < FI < FF for reinnervated yet there is considerable overlap between types as seen clearly in Figure 3.7 A where normal motor unit tetanic force is plotted against fatigue index. The smallest force developing units are fatigue resistant and the largest ones are fatigue sensitive with the Fint units in the intermediate range. Thus fatigability is directly correlated with force as shown in Figure 3.7 B and C for the whole unit and glycogen depleted unit populations, respectively. Although it is not indicated on this graph, the units that sagged occupy the larger half of the force range thus they are both faster and larger.

Figure 3.6. Distribution of tetanic force developed by each of FF, FI, FR, and S motor units in normal and reinnervated muscle. The abcissa is logarithmic indicating that a skewed distribution is present within each type. Mean values are indicated by the arrows and show that S = FR < FI < FF for normal and S < FR < FI < FF for reinnervated muscle.

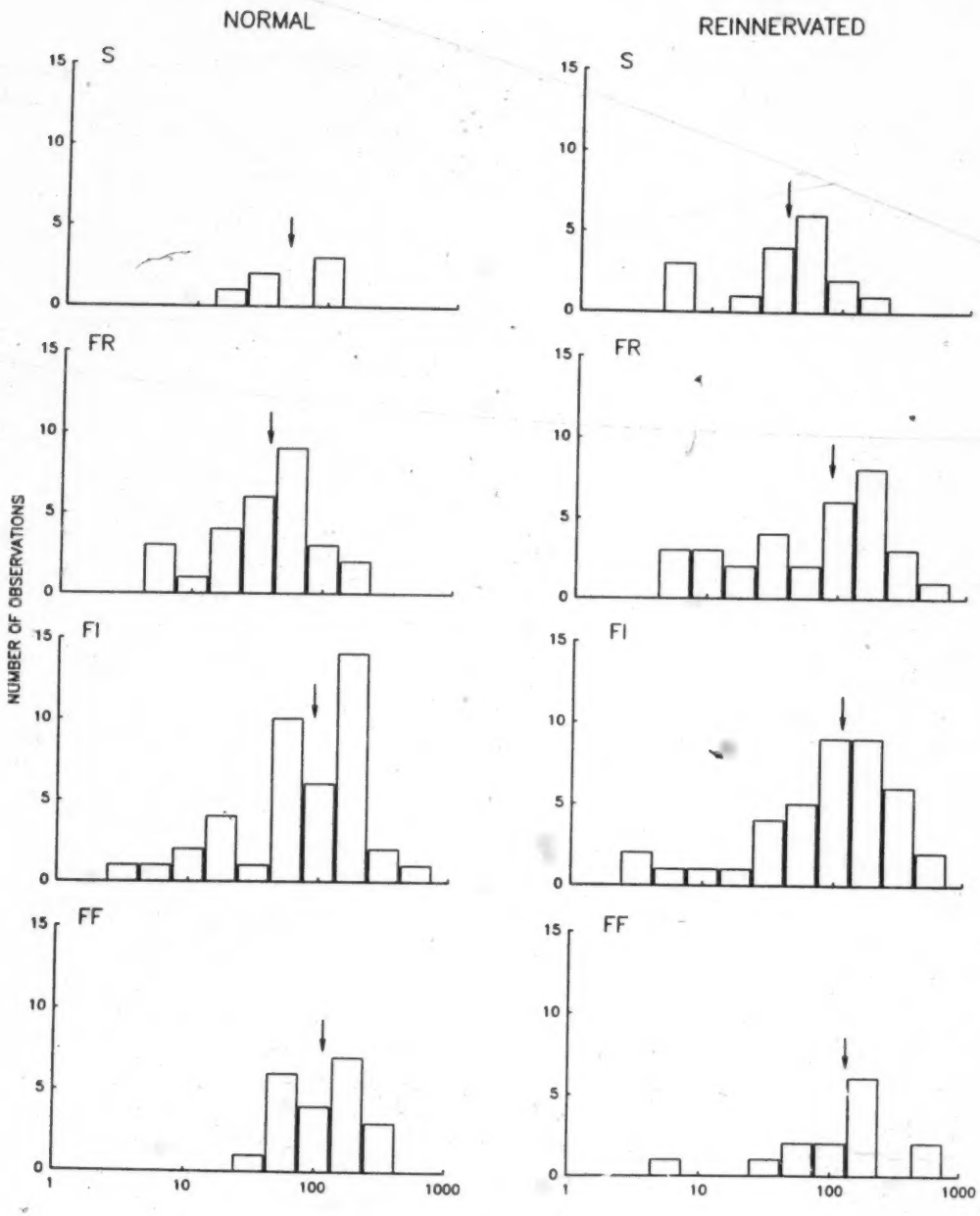
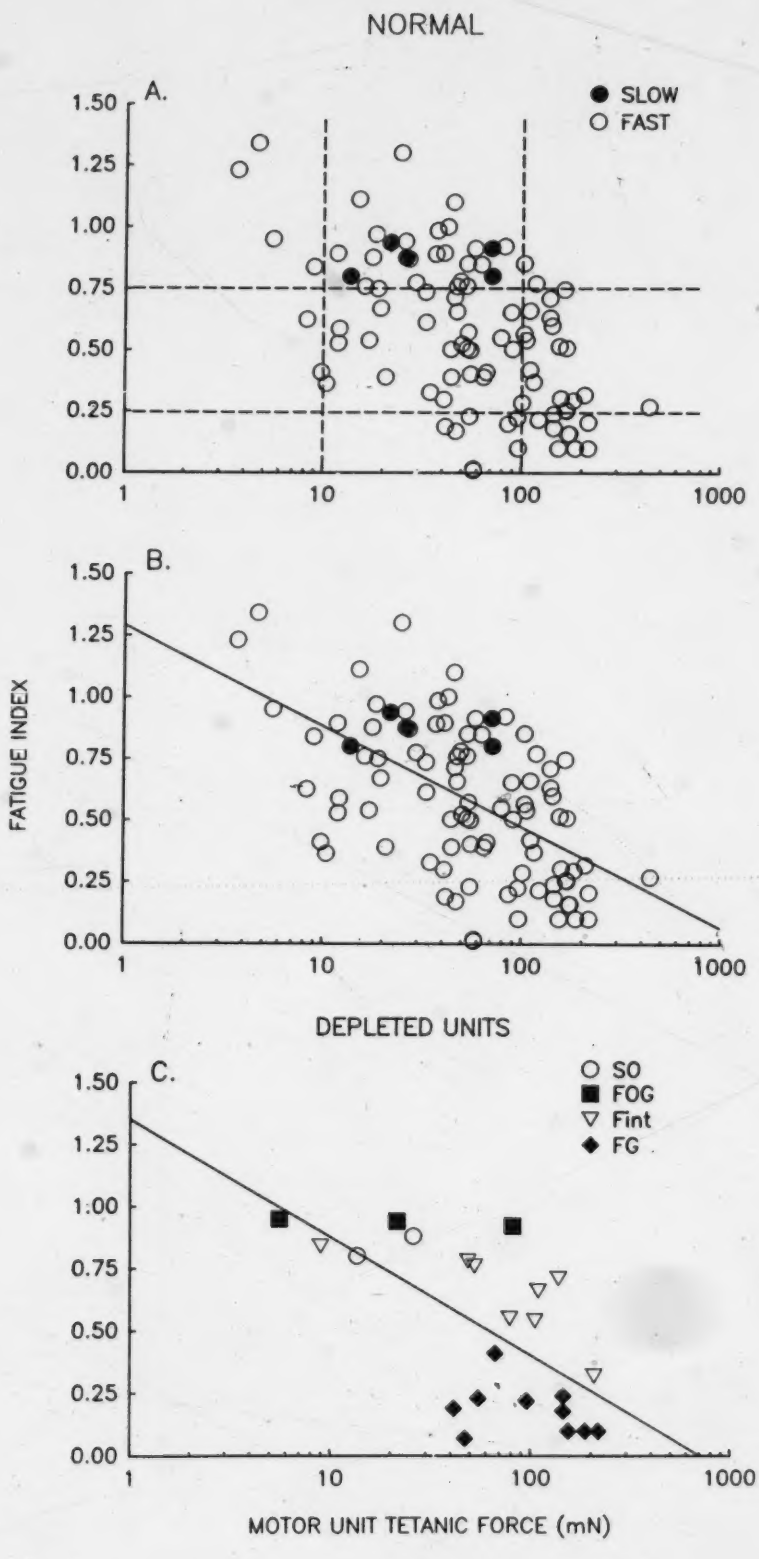

NORMAL
REINNERVATED
S
FR
FI
FF
NUMBER OF OBSERVATIONS
0
5
10
15
1
10
100
1000
MOTOR UNIT TETANIC FORCE (mN)

Figure 3.7. Relationship between tetanic force and fatigue index in normal motor units. (A) The whole population of sampled units is plotted in this graph. Horizontal dashed lines indicate the borders between FF and FI at 0.25 and between FI and FR at 0.75. Vertical dashed lines at 10 and 100 mN indicate that a large proportion of units develop force within this 10-fold range. Slow contracting units with a TTP > 22 ms are shown by filled symbols while the fast contracting units are open symbols. The most fatigue resistant units generally develop small forces while the most fatigue sensitive units develop the largest forces. (B). The same population as in A but with a regression line fitted to the data clearly indicating that units become more fatigable as their force output increases. The slope and standard error of the line is -0.38 ± 0.06, and the correlation coefficient is 0.54. A total of 93 units are sampled in A and B. (C). Only the glycogen depleted units are shown here with their histochemical types denoted by different symbols: SO = open circle, FOG = filled square, Fint = open inverted triangle, and FG = filled diamond. The slope ± standard error of this regression line is -0.48 ± 0.14 and the correlation coefficient is 0.62.


NORMAL
A.
SLOW
FAST
1.50
1.25
1.00
0.75
0.50
0.25
0.00
1
10
100
1000
B.
FATIGUE INDEX
DEPLETED UNITS
C.
SO
FOG
Fint
FG
MOTOR UNIT TETANIC FORCE (mN)

In contrast to normal muscle, the reinnervated unit types span almost the entire force range and the entire range of fatigability (see also Gordon and Stein, 1982b; Gordon et al., 1987; Gordon et al., 1988) and the normal correlation between force and fatigability is lost (Figure 3.8). The slopes of the regression lines fitted to these data (not shown) were not significantly different from zero ($m=-0.10 \pm 0.06$; $r=0.17$ in A, and $m=-0.13 \pm 0.13$; $r=0.19$ in B). Thus it appears that although the reinnervated motor units recover the normal range of force, they do not recover the normal ordering of fatigability which suggests that control over metabolic properties by the reinnervating motoneurone is quite limited.

### 3.III.4 Indirect estimate of IR and ST

Indirect estimates of innervation ratio (IR), and specific tension (ST) require knowledge of the total number of muscle fibres and motor units in a given muscle. Table 3.1 shows that the estimated total number of fibres is 10,060 in normal and 9,390 in reinnervated muscle (line 5); see also Methods) and compares well with an earlier estimate by Edstrom and Kugelberg (1968).

### 3.III.5(a) Innervation Ratios

Indirect estimates of IR from normal muscle are shown in Table 3.2 A. Line 1) gives the total number of muscle fibres which was calculated in the previous

Figure 3.8. Relationship between tetanic force and fatigue index in reinnervated motor units. (A) The whole population of sampled units is plotted here and the dashed lines are the same as in the previous graph. Slow contracting units with a TTP > 22 ms are shown by filled symbols while the fast contracting units are open symbols. In contrast to the normal units in the previous graph, the majority of units are not found between the 10 to 100 mN force range and those with the largest forces have a wider range of fatigability. The regression line fitted to these data (not shown) was not significantly different from zero indicating that the normal trend of decreasing fatigue resistance with increasing force is lost after reinnervation. (B). Only the glycogen depleted units are shown here with their histochemical types denoted by different symbols: SO = open circle, FOG = filled square, Fint = open inverted triangle and FG = filled diamond.

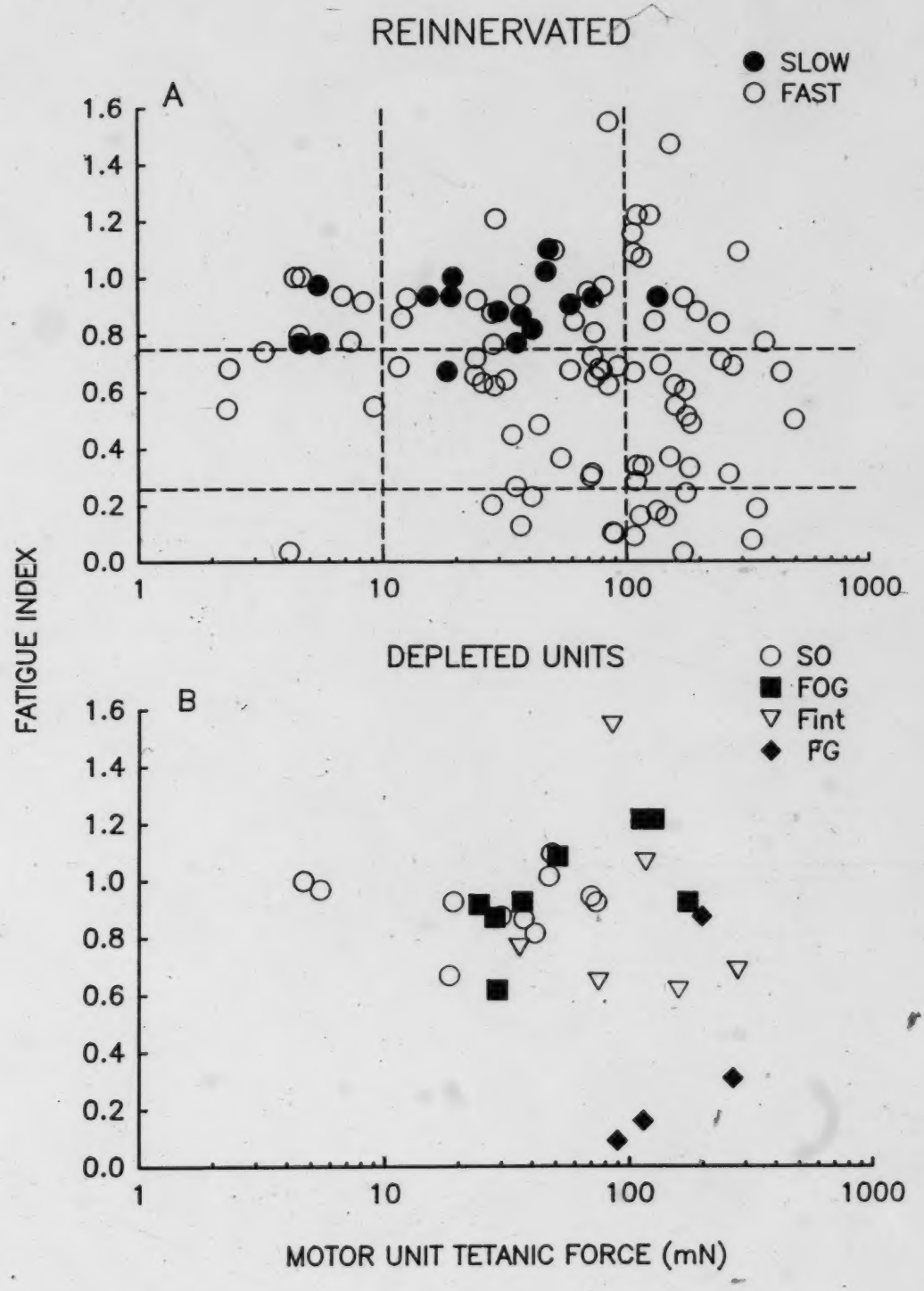

REINNERVATED
SLOW
FAST
A
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
10
100
1000
DEPLETED UNITS
SO
FOG
Fint
FG
B
FATIGUE INDEX
MOTOR UNIT TETANIC FORCE (mN)

TABLE 3.1 ESTIMATED TOTAL NUMBER OF MUSCLE FIBRES IN THE RAT TA MUSCLE.

| | | NORMAL | REINNERVATED |
|---|---|---|---|
| 1) | total CSA of muscle ($mm^2$)[a] | 44.9 ± 1.8 (20) | 40.1 ± 1.3 (20) |
| 2) | total CSA of constant field ($mm^2$) | 0.73 | 0.73 |
| 3) | mean # cells/constant field | 163.2 ± 1.5 (24) | 170.6 ± 2.3 (23) |
| 4) | mean density (# cells/$mm^2$) [3)/2)] | 224 | 234 |
| 5) | total # cells/muscle [4) x 1)] | 10,060 | 9,390 |
| 6) | Estimate of Edstrom & Kugelberg (1968) | 9,325 | |

[a] values are mean ± standard error, number in parentheses is the number of cross-sections measured.

table. The total number of motor units determined by HRP counts (Peyronnard et al., 1986, line 2) agrees well with the estimate obtained from dividing whole muscle force by mean unit force (line 3). The proportion of fibre types (line 4) and of motor unit types (line 6) were obtained as described in the methods. Average IR obtained indirectly (line 8) and directly (line 9) corresponds quite well for type S/SO and FR/FOG units whereas for the other two unit types this is not the case. This can be explained by the fact that the proportions of FI and FF motor unit and FG and Fint muscle fibre types are not well matched. Previous studies have combined the FI and FF types into one category and if this is done here, the estimated IR becomes 127 which agrees well with the directly measured fibre number from FG muscle units.

In Table 3.2 B estimates obtained from reinnervated muscle are shown. In this case, the total number of motor units estimated by HRP (line 2) is higher than the estimate from whole muscle and mean unit force (line 3) which indicates that fewer motoneurones than normal have successfully reinnervated the muscle. For the subsequent calculations the estimated value from line 3) has been used and there is good agreement between indirect (line 8) and direct (line 9) estimates of IR for all unit types except Fint which is again underestimated. In this case, the underestimate cannot be explained by a difference between physiological and histochemical proportions. Note that the mean IR value reported on line 9) for Fint units is associated with a large standard error indicating a high variability in

fibre number. Additionally, Fint units span the whole force range as illustrated in Figure 3.6 and show a large variation in mean fibre area (see Table 2.1 A and B in the previous chapter).

As a further comparison, IR was also estimated using the proportions obtained from the glycogen depleted motor unit data and these values are given in Tables 3.3 A and B. Generated from the normal unit data in A, estimated IR on line 8) agrees quite well with the directly measured fibre number (line 9) and corresponds to force production (line 10) for each unit type. In B, estimated IR from reinnervated depleted units corresponds less well, and the greatest discrepancies are with FR and FF types. These two unit types also have the greatest differences between histochemical and physiological proportions.

### 3.III.5(b) Specific Tension

In Table 3.4 specific tension has been calculated using the mean values of tetanic force and mean total cross-sectional area from the glycogen depleted units. In contrast to previous indirect measurements, specific tension of the slow units is not less than that of fast units and the differences between indirect (line 5) and direct (line 6) estimates can be attributed to the inaccuracies in indirect estimations of IR.

Table 3.2 INDIRECT ESTIMATES OF INNERVATION RATIOS IN MOTOR UNITS OF DIFFERENT TYPE

A. NORMAL

| | | |
|---|---|---|
| 1) | Total # muscle fibres[a] | 10,060 |
| 2) | Total # motor units (MU)[b] | 87 |
| 3) | Whole muscle force/unit force | 84 |

| | | SO | FOG | FInt | FG |
|---|---|---|---|---|---|
| 4) | % fibre types[c] | 3.3 ± 0.7 | 24.1 ± 4.4 | 23.6 ± 0.6 | 49.8 ± 3.7 |
| | n | 153 | 1154 | 1137 | 2371 |
| 5) | total # of each fibre type [3) x 1)] | 332 | 2424 | 2374 | 5010 |
| | | S | FR | FI | FF |
| 6) | % MU types | 6.1 | 27.6 | 44.9 | 21.4 |
| | n | 6 | 27 | 44 | 21 |
| 7) | total # of each MU type [5) x 2)] | 5 | 24 | 39 | 19 |
| 8) | average IR [4)/6)] | 66 | 101 | 61 | 264 |
| 9) | actual measured IR[d] | 57.5 ± 0.4 | 106.5 ± 7.4 | 145.7 ± 18.1 | 122.2 ± 10.6 |
| | n | 2 | 2 | 6 | 10 |
| 10) | Tetanic Force (mN)[d] | 49.2 ± 28.5 | 35.1 ± 4.7 | 96.1 ± 15.2 | 99.9 ± 12.6 |

[a]estimated as shown in Table 3.1 (See also Methods)

[b]from Peyronnard et al. (1986)

[c]values are mean ± standard error

[d]units are grouped according to histochemical type

Table 3.2 INDIRECT ESTIMATES OF INNERVATION RATIOS IN MOTOR UNITS OF DIFFERENT TYPE

B. REINNERVATED

| | | | | |
|---|---|---|---|---|
| 1) Total # muscle fibres[a] | 9,390 | | | |
| 2) Total # motor units (MU)[b] | 87 | | | |
| 3) Whole muscle force/unit force | 68 | | | |
| | SO | FOG | Fint | FG |
| 4) % fibre types[c] | 14.4 ± 3.4 | 26.8 ± 2.4 | 36.9 ± 3.4 | 21.9 ± 2.3 |
| n | 628 | 1509 | 1903 | 1227 |
| 5) total # of each fibre type [3) x 1)] | 1352 | 2516 | 3465 | 2056 |
| | S | FR | FI | FF |
| 6) % MU types | 20.2 | 29.4 | 37.6 | 12.8 |
| n | 22 | 32 | 41 | 14 |
| 7) total # of each MU type [5) x 2)] | 14 | 20 | 25 | 9 |
| 8) average IR [4)/6)] | 96 | 126 | 139 | 228 |
| 9) actual measured IR[d] | 93.3 ± 17.4 | 129.9 ± 23.5 | 198.2 ± 38.8 | 197.0 ± 19.5 |
| n | 4 | 7 | 6 | 3 |
| 10) Tetanic Force (mN)[d] | 45.0 ± 8.4 | 104.2 ± 20.3 | 123.6 ± 25.7 | 129.5 ± 26.4 |

[a]estimated as shown in Table 3.1 (See also Methods)

[b]from Peyronnard et al. (1986)

[c]values are mean ± standard error

[d]units are grouped according to histochemical type

Table 3.3 INDIRECT ESTIMATES OF INNERVATION RATIOS CALCULATED FROM THE GLYCOGEN DEPLETED MOTOR UNIT DATA

A. NORMAL

| | |
|---|---|
| 1) Total # muscle fibres[a] | 10,060 |
| 2) Total # motor units (MU)[b] | 87 |
| 3) Whole muscle force/unit force | 84 |

| | SO | FOG | Fint | FG |
|---|---|---|---|---|
| 4) % fibre types | 10 | 10 | 30 | 50 |
| n | 2 | 2 | 6 | 10 |
| 5) total # of each fibre type | 1006 | 1006 | 3018 | 5030 |

| | S | FR | FI | FF |
|---|---|---|---|---|
| 6) % MU types | 15 | 15 | 25 | 45 |
| n | 3 | 3 | 5 | 9 |
| 7) total # of each MU type | 13 | 13 | 22 | 39 |
| 8) average IR [4)/6)] | 77 | 77 | 137 | 129 |
| 9) actual measured IR[c] | 57.5 ± 0.4 | 106.5 ± 7.4 | 145.7 ± 18.1 | 122.2 ± 10.6 |
| n | 2 | 2 | 6 | 10 |
| 10) Tetanic Force (mN) | 20.1 ± 4.3 | 52.0 ± 21.4 | 106.3 ± 22.5 | 115.8 ± 18.8 |

[a]estimated as described in the methods

[b]from Peyronnard et al. (1986)

[c]values are mean ± standard error

Table 3.3 INDIRECT ESTIMATES OF INNERVATION RATIOS CALCULATED FROM THE GLYCOGEN DEPLETED MOTOR UNIT DATA

B. REINNERVATED

| | |
|---|---|
| 1) Total # muscle fibres[a] | 9390 |
| 2) Total # motor units (MU)[b] | 87 |
| 3) Whole muscle force/unit force | 68 |

| | SO | FOG | Fint | FG |
|---|---|---|---|---|
| 4) % fibre types | 20 | 35 | 30 | 15 |
| n | 4 | 7 | 6 | 3 |
| 5) total # of each fibre type | 1878 | 3287 | 2817 | 1409 |

| | S | FR | FI | FF |
|---|---|---|---|---|
| 6) % MU types | 25 | 50 | 20 | 5 |
| n | 5 | 10 | 4 | 1 |
| 7) total # of each MU type | 17 | 34 | 14 | 3 |
| 8) average IR [(4)/(6)] | 110 | 97 | 201 | 469 |
| 9) actual measured IR[c] | 93.3 ± 17.4 | 129.9 ± 23.5 | 198.2 ± 38.8 | 197.0 ± 19.5 |
| n | 4 | 7 | 6 | 3 |
| 10) Tetanic Force (mN) | 49.9 ± 7.8 | 79.2 ± 20.4 | 125.1 ± 31.9 | 192.9 ± 35.9 |

[a]estimated as described in the methods

[b]from Peyronnard et al. (1986)

[c]values are mean ± standard error

Table 3.4 INDIRECT ESTIMATES OF SPECIFIC TENSION

| | NORMAL | | | | REINNERVATED | | | |
|---|---|---|---|---|---|---|---|---|
| | SO | FOG | Fint | FG | SO | FOG | Fint | FG |
| 1) Mean CSA | 1317.2 | 2047.9 | 3269.7 | 4134.4 | 2252.3 | 2424.3 | 2537.7 | 3896.7 |
| 2) IR[a] | 77 | 77 | 137 | 129 | 110 | 97 | 201 | 469 |
| 3) Total CSA ($cm^2 \times 10^{-3}$) | 1.01 | 1.58 | 4.48 | 5.33 | 2.48 | 2.35 | 5.10 | 18.27 |
| 4) Mean Tetanic Force (g) | 2.05 | 5.31 | 10.08 | 11.82 | 5.09 | 8.08 | 12.76 | 19.68 |
| 5) Specific Tension[b] | 2.03 | 3.36 | 2.41 | 2.22 | 2.05 | 3.44 | 2.50 | 1.08 |
| 6) Specific Tension[c] | 2.63 | 2.21 | 2.42 | 2.29 | 2.57 | 2.36 | 2.52 | 2.63 |

---

a average IR taken from line 8) in Table 3.3 A and B
b calculated from indirect estimates of IR; units are ($kg/cm^2$)
c calculated from direct fibre counts

## 3.IV. DISCUSSION

The major findings from this study are: 1) there is essentially a continuum of properties in normal muscle such that unit classification creates divisions with considerable overlap between types, particularly with respect to the more fatigue resistant units, 2) Normally force is well correlated with fatigue resistance but force and fatigability are dissociated after reinnervation, 3) Comparison of direct and indirect methods show that a mismatch between physiological and histochemical classification of units can lead to considerable errors in indirect estimations of IR which get compounded in the estimates of specific tension, 4) The number of muscle fibres per motoneurone (IR) increases to compensate for the reduction in the number of nerves which successfully get back to the muscle. Thus, size dependent branching and expansion is an important mechanism leading to the successful recovery of reinnervated muscles.

### 3.IV.1 Motor Unit Types or Continuum?

#### 3.IV.1(a) Speed

In many muscles, including the rat TA in the present study, neither contraction time nor sag can separate all motor units into fast or slow categories unambiguously (Reinking et al., 1975; Goslow et al., 1977; McDonagh et al.,

1980; Fleshman et al., 1981; Jami et al., 1982; Gillespie et al., 1987). The histochemical reactions for myosin ATPase that typically result in three staining intensities, depend critically on pH sensitivities which vary between animals and may also vary between muscles within the same animal (Brooke and Kaiser, 1974). The existence of a variety of both myosin and tropomyosin isoforms in a variety of combinations (Staron and Pette, 1987) and the observation that antibodies to both fast and slow myosin cross-react in certain rat soleus fibres (Gauthier and Lowey, 1979) reinforce the concept that the biochemical properties associated with contraction speed of muscle fibres represent a continuum as opposed to discrete types.

**3.IV.1(b) Fatigability**

Units in this study have been classified with Burke's method, the progressive fatigue index (PFI), which expresses the peak force attained after 2 minutes of stimulation relative to that at the start of stimulation (Burke et al., 1973). These data show a continuous distribution over the entire range of fatigability which corresponds well with results from a different technique of progressive fatigue over 4 minutes (Kugelberg and Lindegren, 1979). This latter method is an adaptation of the cumulative fatigue index (CFI) which expresses the force accumulated during the first two minutes relative to that accumulated during the entire four minutes of stimulation (Reinking et al., 1975). The PFI and CFI do not always yield the same data when applied to muscles containing very few FI units

(MG: Burke et al., 1973 vs. Reinking et al., 1975; see also Botterman and Cope, 1988 for a third approach). On the other hand, in muscles with a higher proportion of FI units (TP: McDonagh et al., 1980; PL: Kernell et al., 1983), similar results were obtained from both methods. Thus it appears that the two methods agree when used on muscles with a relatively high proportion of FI units and that the CFI 'brings out' more intermediate units in muscles where there are few (MG). The MG muscle has been the most extensively studied and is perhaps the most extreme example of a bimodal distribution.

Resistance to fatigue is a labile property determined by the proportions of oxidative and glycolytic enzymes, which are readily changed by an altered pattern of usage (Burke and Edgerton, 1975; Pette and Vrbova, 1985; Edstrom and Grimby, 1986). Recent microanalytical analysis of single fibres has shown a broad range of metabolic enzyme activities within both fast (type II) and slow (type I) fibre types such that both groups represent metabolically heterogeneous populations (Pette and Spamer, 1986). Although type I and type II fibres have essentially non-overlapping ranges in glycolytic enxyme activities, the oxidative enzyme activities show extensive overlap. Thus the type II fibres exhibit a continuous spectrum of metabolic properties and as suggested by Pette and Spamer (1986) the FOG and FG types determined qualitatively may represent extremes of this spectrum.

### 3.IV.2 Dissociation of Force and Fatigability

After reinnervation a similar range of force and metabolic properties is re-established but the correlation between them is lost. This is consistent with previous findings from reinnervated muscle (Gordon and Stein, 1982b; Gillespie et al., 1987; Gordon et al., 1988; Cope and Botterman, 1987) but is even more obvious because of the good correlation in normal rat TA muscle (see Figure 3.7B). Reinnervating motoneurones supply a diverse population of muscle fibres that formerly belonged to several different motor units and it is likely that there is an increased metabolic heterogeneity within the newly formed units. Although nerves can influence the expression of myosin in the muscle fibres they reinnervate to result in uniformity of histochemical type (Gauthier et al., 1985), the pattern of activation (or functional usage) evidently cannot totally respecify the metabolic properties of these fibres or even their cross-sectional areas (Totosy de Zepetnek et al., 1988). It is possible that, as suggested by Westgaard and Lomo (1988), individual muscle fibres possess an adaptive range beyond which they cannot be altered.

Considering the above evidence, it would appear that a continuum exists in the histochemical as well as physiological properties of muscle fibres within a given muscle. Each muscle contains fibres with a certain spectrum of metabolic enzyme activities and contractile properties determined by its functional usage.

Thus, in some muscles different subtypes of motor units are readily apparent from the clustering of various mechanical properties but in others the distribution of properties appears continuous. The common factor in all studies however, regardless of how fatigue resistance is assessed or whether clustering is apparent or not, is that fatigue resistance decreases as force increases.

### 3.IV.3 Indirect and Direct Estimates of IR

Estimated IRs for type S units from a number of different indirect studies were considerably higher than would be expected from their force production and average fibre size (shown in Table 2.2 of the preceding chapter). Additionally, the estimates for S units were not very different from FF units which develop considerably greater forces. This was not seen in the present study. The indirect estimates were closer to the actual measured values of IR than expected and varied more closely with force than the previously reported results using this approach. The notable exceptions outlined in the results could be explained in most cases by differences in the physiological and histochemical proportions of types.

The possible sources of error may involve the numerator (proportion of muscle fibres) and/or the denominator (proportion of motor units) in the equation used to estimate the relative innervation ratio (RIR) for each unit type. Errors in

the numerator may arise from inadequate sampling in muscles that are highly regionalized with respect to the occurrence of different fibre types and from the inability to distinguish between Fint and FG types. Localized sampling in these muscles could bias the estimated proportions of fibre types. Errors in the denominator could again arise from unrepresentative sampling or from problems in classification of the motor unit population. For example, if some units defined as FR physiologically are SO histochemically, as they are in Figure 3.5, then the proportion of slow motor units will be under-represented leading to an overestimated RIR. Conversely, if there are fewer muscle units than motor units of the corresponding type then RIR will be underestimated. A further source of error involves estimations of the total number of fibres in the muscle and the total number of motor units supplying the muscle which are used to calculate the total muscle fibre number and total motor unit number of each type. Each of these errors become additive in the calculations leading to a much larger error in the final estimate of IR which then becomes incorporated into the estimate of specific tension.

Indirect estimates of specific tension from the glycogen depleted motor unit data agree reasonably well with the direct measurements despite errors of classification and the increased overlap in properties of reinnervated units. All estimates, with the exception of that for FG units in reinnervated muscle (Table 3.4), are within the range of previously reported values (see Chapter 2, Table

2.3). The low value for FG units can be accounted for by the mismatch in proportions which led to a large overestimate of IR. Yet the major differences between previous direct and indirect values occurred with the SO population which in normal rat TA muscle represents only 3-5% of the total population. Furthermore, the values used for total cross-sectional area (the product of fibre number and mean unit fibre area) in these calculations were obtained directly from the same population of units from which force and fibre number were measured. If fibre areas of each type were taken randomly from the muscle the errors would likely be greater. Ultimately, there are a large number of possible errors involved in the indirect estimates of IR and specific tension which, when added together, are likely greater than the errors involved in the direct determinations of fibre number.

The physiological results from this study have shown that motor unit properties in the TA muscle of the rat, including contraction speed, fatigability, and force, as well as the number and size of muscle fibres per unit, represent a continuum. Differentiation of motor units into distinct types using well established criteria showed considerable overlap between types in normal muscles and even more overlap in reinnervated muscle. This continuum is not as striking in cat limb muscles where there are fewer FI units and less overlap between types.

# CHAPTER 4

# COMPARISON OF FORCE AND EMG MEASURES

## 4.1 INTRODUCTION

The magnitude of the electromyographic (EMG) potential from an active motor unit is related to the magnitude of the mechanical output (force) of that motor unit. Small, low threshold motor units (those first activated) generally have small EMG spikes and produce small forces (Henneman et al., 1965a; Milner-Brown et al., 1975). For the whole muscle, as tension increases, the EMG wave tends to increase but the exact relation between muscle electrical activity and tension is highly complex. It has been suggested that the amplitude of summed potentials should be proportional to the number of active fibres (Katz, 1966; Kosarov et al., 1974) and theoretical studies have predicted that surface EMG amplitude should increase as the square root of muscle force (Moore, 1967). In support of the theoretical prediction, Milner-Brown and Stein (1975) showed in human hand muscle that the surface EMG amplitude from individual motor units increased approximately as the square root of twitch tension. The situation is more complex when considering the whole muscle. The relation between force and EMG as the population of active motor units increases depends on the

relative contributions of recruitment and rate coding, as well as on the degree of overlap of unit potentials.

Needle electrodes have been commonly used to estimate electrical and spatial characteristics of normal motor units as well as those subjected to injury or disease (Buchthal et al, 1957; McComas et al., 1971; Stalberg and Thiele, 1975). Only a portion of the muscle unit fibres are sampled with these electrodes and the recorded voltage is critically dependent on the position of the needle. Surface EMG recordings represent electrical activity from all or most of the fibres (provided the electrode is large enough relative to the size of the muscle) and are less dependent on the position of the electrode. As described by Kosarov et al. (1974) the summated surface potential (P) of the active fibres in a motor unit decreases in amplitude with the inverse of the radial distance between the electric axis of the motor unit and the recording electrode (r) according to the equation:

$$P = \frac{a}{r^n} \qquad (1)$$

where a represents the size of the motor unit and n is a constant determined experimentally. One would expect that for units of comparable force, those lying deep in the muscle would contribute less to the surface EMG than superficial

units. However Milner-Brown and Stein (1975) did not observe such a decrement with depth in the human hand muscle and suggested that the unit fibres in this muscle may be scattered over a wide range of depths.

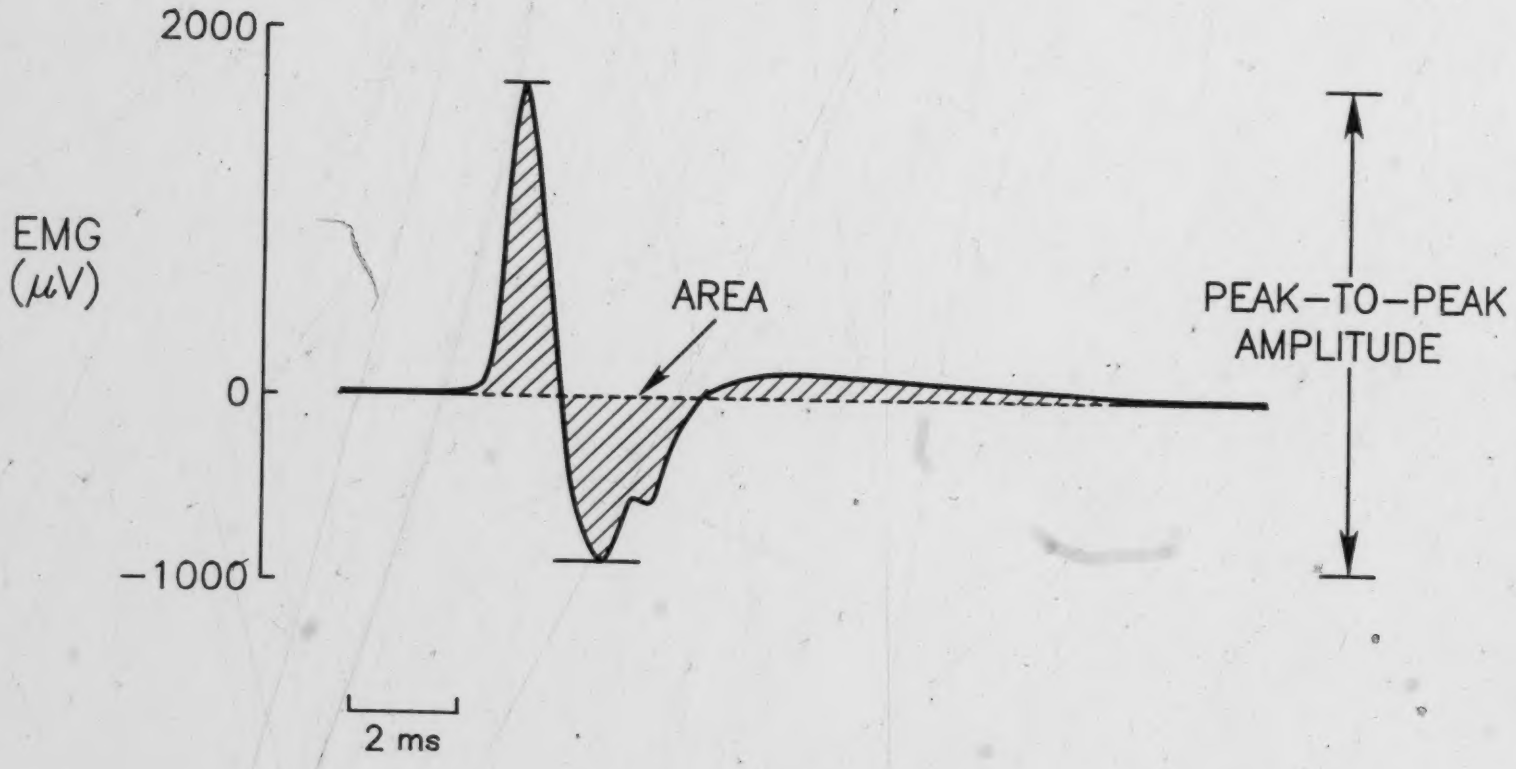
The size and configuration of the myoelectric signal from an active motor unit depends on a variety of factors including 1) electrode design, 2) electrode placement, 3) the number of muscle fibres innervated (innervation ratio) by the motoneurone, 4) spatial density of the muscle fibres, 5) average muscle fibre size and 6) muscle architecture. In the present study we have examined the influence of some of the above factors on the recorded EMG in single motor units from both normal and reinnervated rat tibialis anterior (TA) muscle. A sample of units in both muscle groups were glycogen depleted allowing the number, size and spatial location of all the constituent fibres to be directly determined. Since muscle fibres in reinnervated motor units are distributed in clumps in contrast to the diffuse scatter of normal muscle units (Kugelberg et al., 1970; see also Figure 2.7) we could investigate whether this altered distribution of fibres alters the normal relation between EMG and force.

## 4.II METHODS

The experimental procedure used for preparation and recording has been described in detail elsewhere (Chapter 2). Briefly, female Sprague-Dawley rats (200-400g) were anaesthetized with 45 mg/kg sodium pentobarbital (somnotol). The spinal cord was exposed by laminectomy from T12 to L6 and the distal tendon of the tibialis anterior muscle was attached to a Grass strain gauge (force range 50-1000g) for isometric force recording. All other hindlimb muscles were denervated by either cutting or crushing their nerve supply. Under the miscroscope ventral roots were dissected into fine filaments until graded stimulation of a filament elicited all-or-none events in both the EMG potential and twitch amplitude indicating the presence of a single motor unit. The electrodes used for recording of EMG potentials were either intramuscular or surface.

Four fine (75 $\mu$m) teflon-coated stainless steel wires bared at the tip were inserted into the muscle in a proximo-distal row to obtain intramuscular EMG. An 8x12 mm silastic sheet was sutured onto the muscle fascia over the middle two-thirds of the muscle for surface EMG. Two rows of stainless steel wire spaced 6 mm apart were coiled through it. Measurements made on the EMG records to obtain amplitude and integrated area (IEMG) values are shown in Figure 4.1. The maximum excursion between negative-going and positive-going phases of the waveform is the peak to peak amplitude in microvolts and the

Figure 4.1. Example of an EMG recording from a single motor unit. Peak to peak amplitude (in microvolts) is measured from the maximum excursion between the positive and negative phases of the waveform and integrated EMG (in microvolt-milliseconds) is measured as the area under the waveform.


2000
EMG
(μV)
0
−1000
AREA
PEAK−TO−PEAK
AMPLITUDE
2 ms

area under the entire waveform is the integrated EMG in microvolt-milliseconds. Small force producing units are associated with small amplitude and relatively long duration EMG signals while the converse is true for large force producing units. EMG and force signals were amplified and then averaged on a PDP-11 computer and stored on disks for later analysis.

Contractile speed and force, as well as an index of fatigue resistance were obtained from each isolated motor unit for physiological characterization and type identification. As described in detail in Chapter 2, the stimulus used for glycogen depletion depended on the physiological characteristics of the unit.

Groups of 8-10 serial cross-sections 10 $\mu$m thick were taken from different proximodistal levels of the muscle and the following histochemical stains were used. Periodic acid-Schiff (PAS) (Pearse, 1960) stains for glycogen and each of the fibres belonging to the stimulated motor unit were identified by their negative staining. In this way, a direct count of total unit fibre number was obtained as well their cross-sectional areas and spatial location. For more details on the techniques used for these measurements see Chapter 2.

## 4.III RESULTS

### 4.III.1 EMG Amplitude and Area from Single Units

We have previously shown (Chapter 2) that motor unit force in both normal and reinnervated muscles varies over approximately the same 100-fold range. In the present study we see that EMG amplitude and area vary over a much wider range, both approaching 1000-fold as seen in Figure 4.2. Note that these distributions are approximately normally distributed on a logarithmic scale indicating that the smaller EMG signals make up the bulk of the population. This is consistent with what we found from motor unit force distributions indicating that mechanical and electrical characteristics of motor units are qualitatively well matched. The cumulative distributions shown in Figure 4.3 show that the reinnervated unit EMG curves are shifted slightly to the right from the normal curves. Since the distribution of force did not differ between these two groups as shown in Chapter 2, this shows that the EMG signal is larger in reinnervated muscles for units of comparable size. Thus the clumping of muscle fibres in these units apparently produces larger EMG signals for a given force than do the scattered fibres in normal muscle.

Plotting the area under the EMG waveform against EMG amplitude shows that in both muscle groups these two parameters are linearly related with an intercept near zero (Figure 4.4). The slope of the fitted lines on the log-log

Figure 4.2 Distributions of EMG amplitude and area in normal and reinnervated motor units. In each of these graphs the distribution extends over a 1000-fold range and is normally distributed on a logarithmic scale. The mean values (shown by the arrows) for EMG amplitude in normal units (A) is 431.0 ± 59.6 $\mu$V (n=129) and for reinnervated units (B) is 678.6 ± 73.8 $\mu$V (n=158) while mean EMG area is 418.1 ± 58.3 $\mu$V.ms (n=123) for normal (C) and 834.4 ± 92.8 $\mu$V.ms (n=156) for reinnervated units (D).

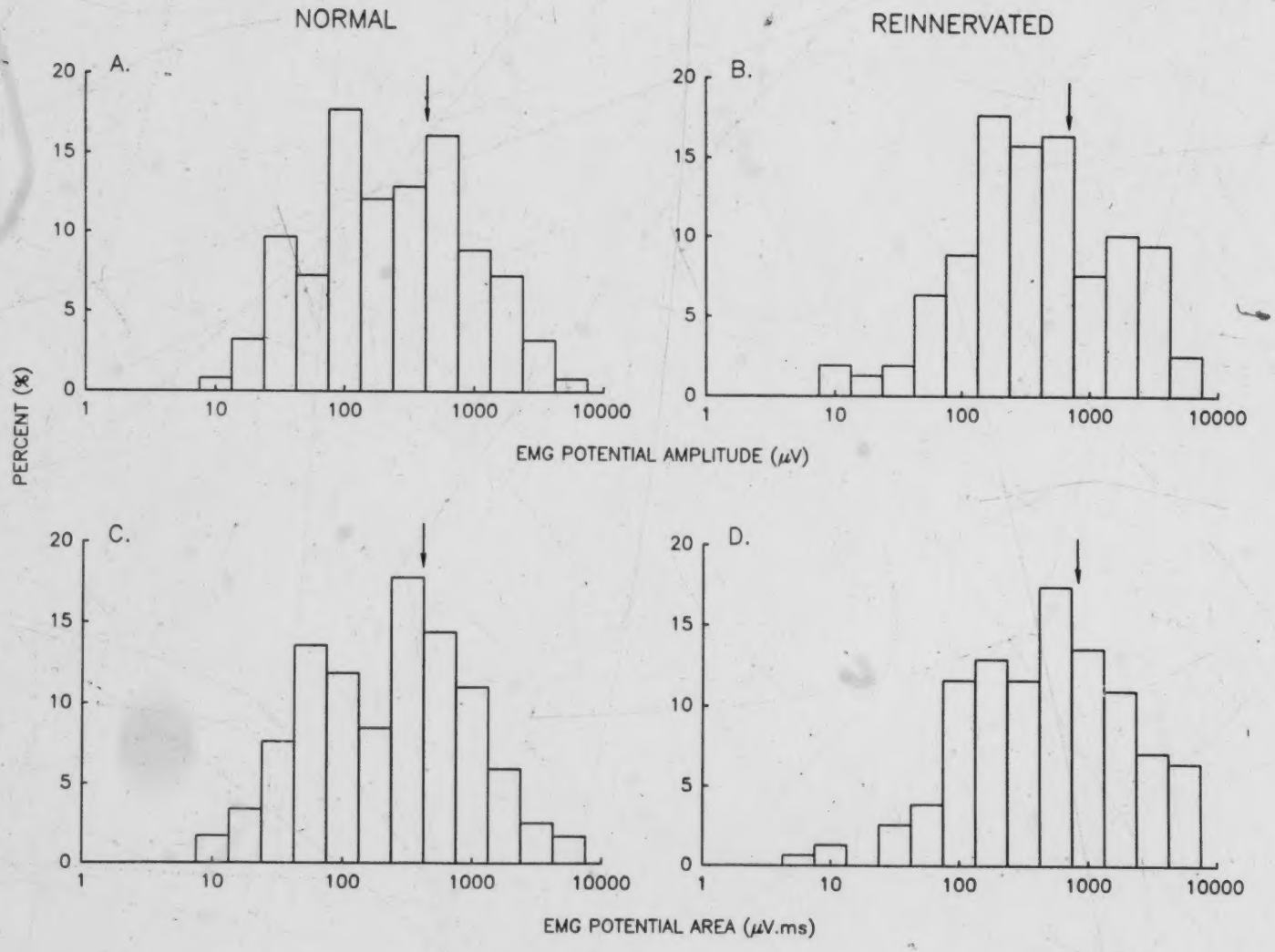
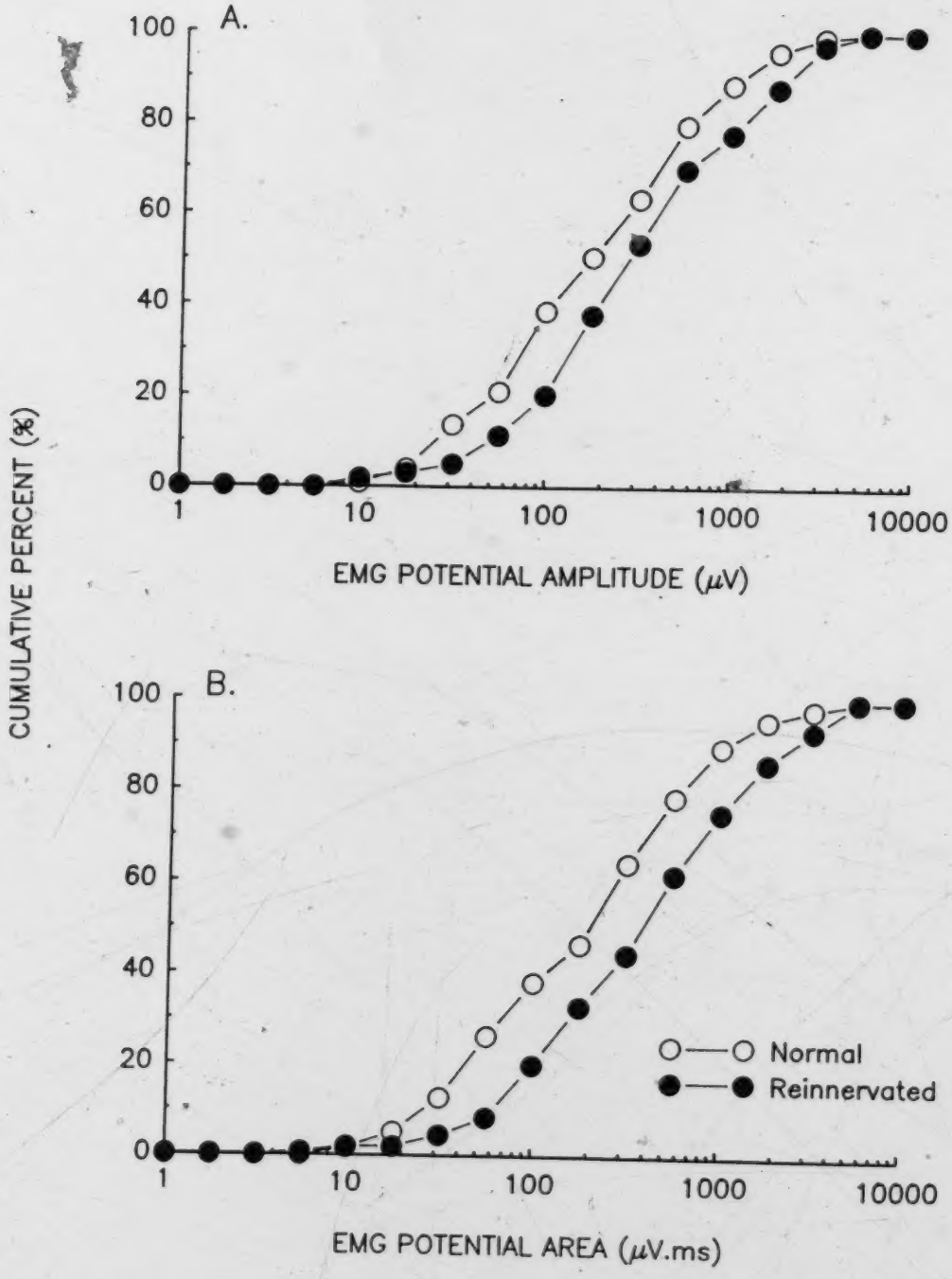

NORMAL
REINNERVATED
A.
B.
C.
D.
PERCENT (%)
EMG POTENTIAL AMPLITUDE (μV)
EMG POTENTIAL AREA (μV.ms)
0
5
10
15
20
1
10
100
1000
10000

Figure 4.3 Cumulative distributions of EMG amplitude and area comparing normal and reinnervated motor units. The distributions of both amplitude and area from reinnervated units show a significant shift to the right (Kolmogorov-Smirnov test, $P < 0.05$) indicating that the EMG signals from reinnervated units are larger than normal.


A.
100
80
60
40
20
0
1
10
100
1000
10000
EMG POTENTIAL AMPLITUDE (μV)
CUMULATIVE PERCENT (%)
B.
100
80
60
40
20
0
1
10
100
1000
10000
EMG POTENTIAL AREA (μV.ms)
Normal
Reinnervated

plots is near unity with values of 0.93 ± 0.02 (r=0.98) and 0.91 ± 0.03 (r=0.94) for normal and reinnervated units, respectively. Thus these two parameter measures of EMG are virtually interchangeable. Milner-Brown and Stein (1975) reported a similar correlation between area and amplitude of EMG potentials recorded from human first dorsal interosseus muscles. Since the intramuscular and surface EMG recordings were pooled in Figure 4.4, it appears that there is no difference between the two electrode configurations, at least with respect to the relation between area and amplitude of the signal.

### 4.III.2 EMG Amplitude and Force

In Figure 4.5 EMG potential amplitude is plotted as a function of twitch force. In A and B the recordings are from intramuscular wire electrodes and in C and D the recordings are from the surface probe. There is considerable scatter in the data (as shown previously by Milner-Brown and Stein, 1975 and Clamann and Robinson, 1985) from both muscle groups. Considering first the data from the wire electrodes, the relationship between EMG amplitude and twitch force is virtually identical in normal and reinnervated muscle. If the integrated EMG potential is plotted against twitch force the result is the same. Considering now the surface EMG recordings in C and D, it is apparent that the relationship is not different from that seen using intramuscular electrodes. Taken together,

Figure 4.4 Relation between EMG amplitude and area. In both normal (A) and reinnervated (B) units EMG area increases linearly with amplitude and the slope of the regression lines is 0.93 ± 0.02 (r=0.98) and 0.91 ± 0.03 (r=0.94) for normal and reinnervated, respectively. These two EMG parameters are virtually interchangable.

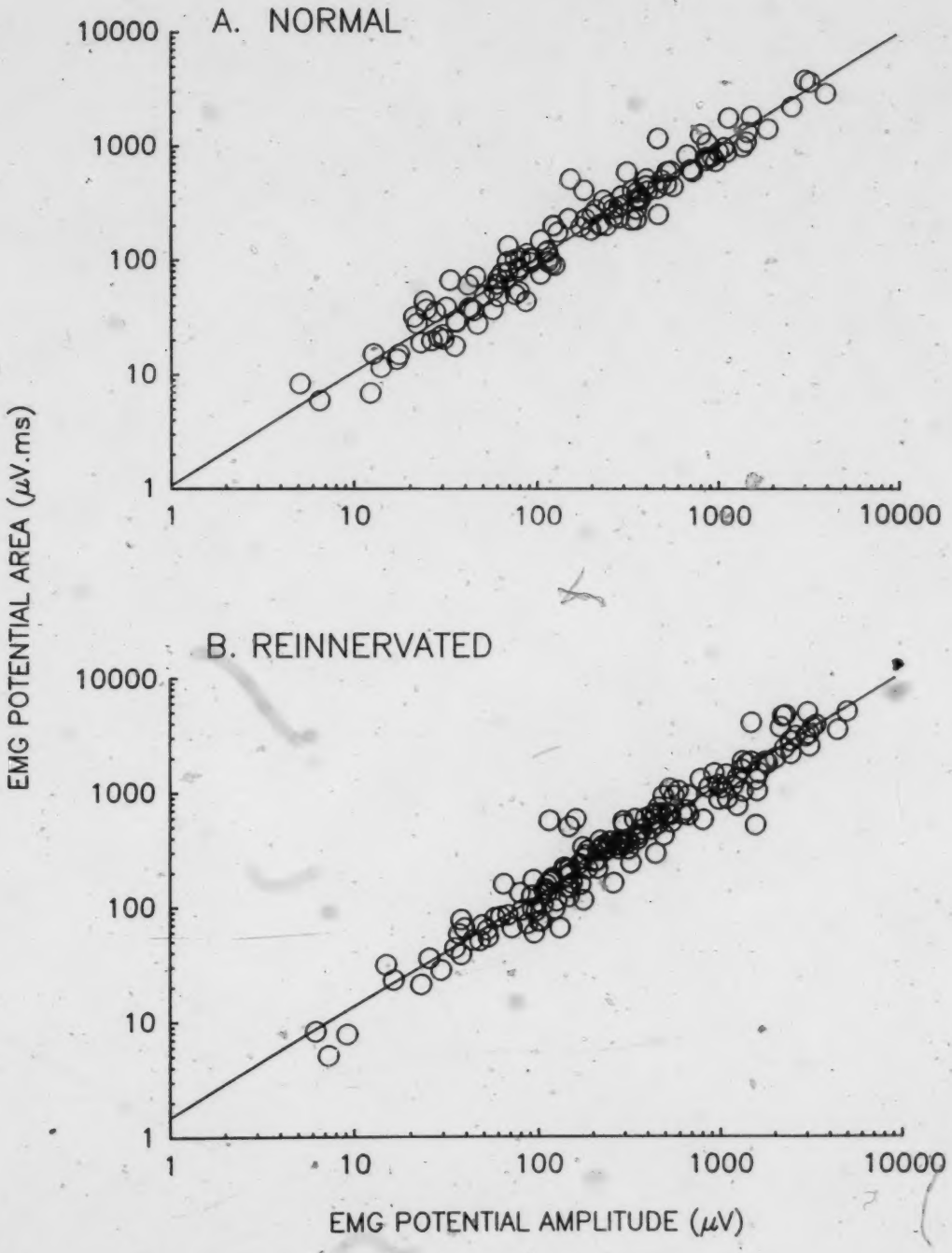
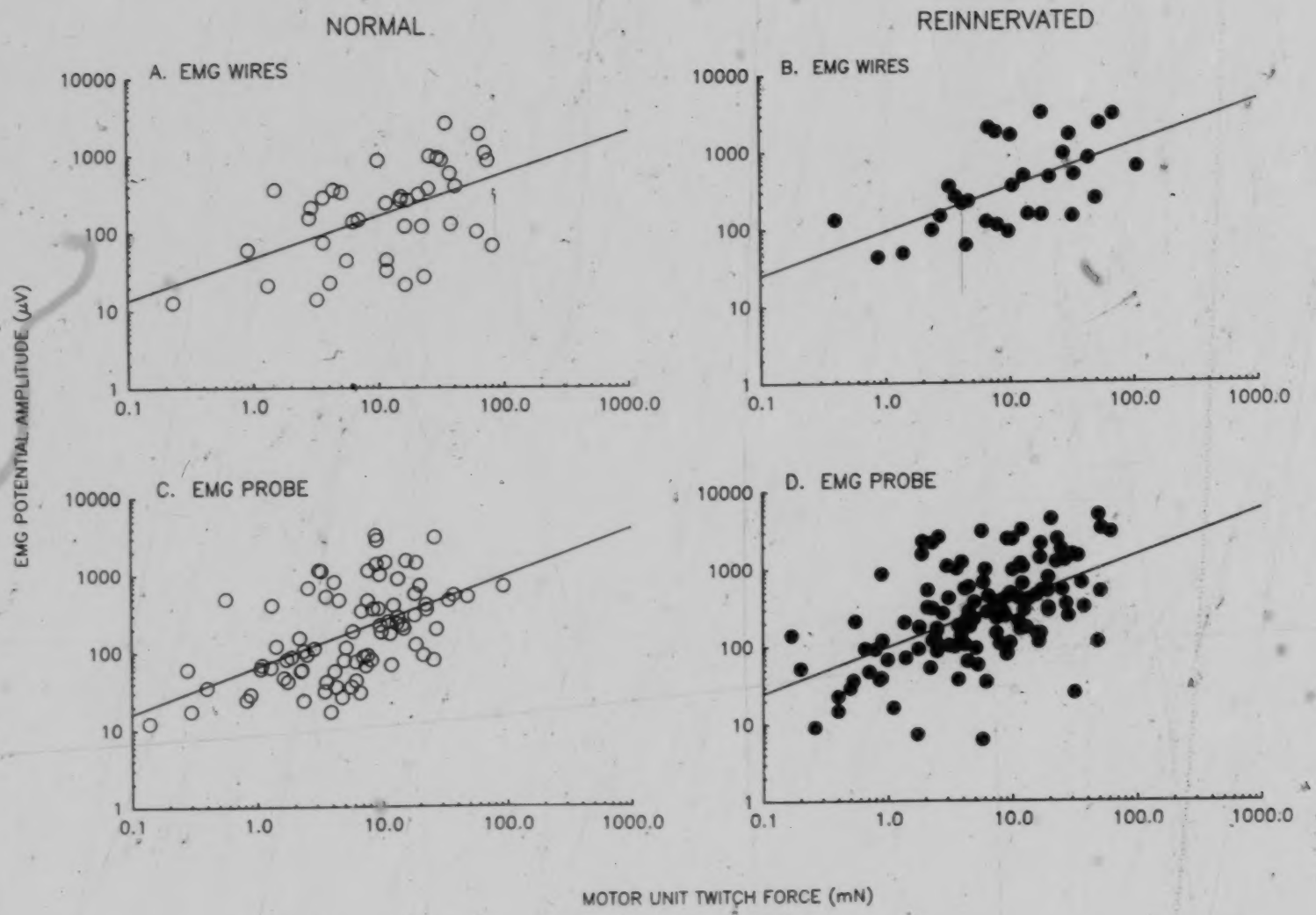

A. NORMAL
B. REINNERVATED
EMG POTENTIAL AREA (μV.ms)
EMG POTENTIAL AMPLITUDE (μV)
10000
1000
100
10
1
1
10
100
1000
10000

Figure 4.5 Comparison of the relation between motor unit twitch force and EMG amplitude recorded with wire electrodes or a surface probe. The EMG signal was recorded from 4 wires inserted into the muscle and the slope of the relation between twitch force and EMG are 0.54 ± 0.14 (n=40) for normal (A) and 0.56 ± 0.14 (n=37) for reinnervated (B) units. In C and D EMG was recorded from a surface probe sewn onto the muscle fascia. The slopes in these graphs are 0.58 ± 0.09 (n=88) and 0.60 ± 0.08 (n=126) for normal and reinnervated, respectively. In all cases the EMG is seen to increase as the square root of force.


NORMAL
REINNERVATED
A. EMG WIRES
B. EMG WIRES
C. EMG PROBE
D. EMG PROBE
EMG POTENTIAL AMPLITUDE (μV)
MOTOR UNIT TWITCH FORCE (mN)
10000
1000
100
10
1
0.1
1.0
10.0
100.0
1000.0

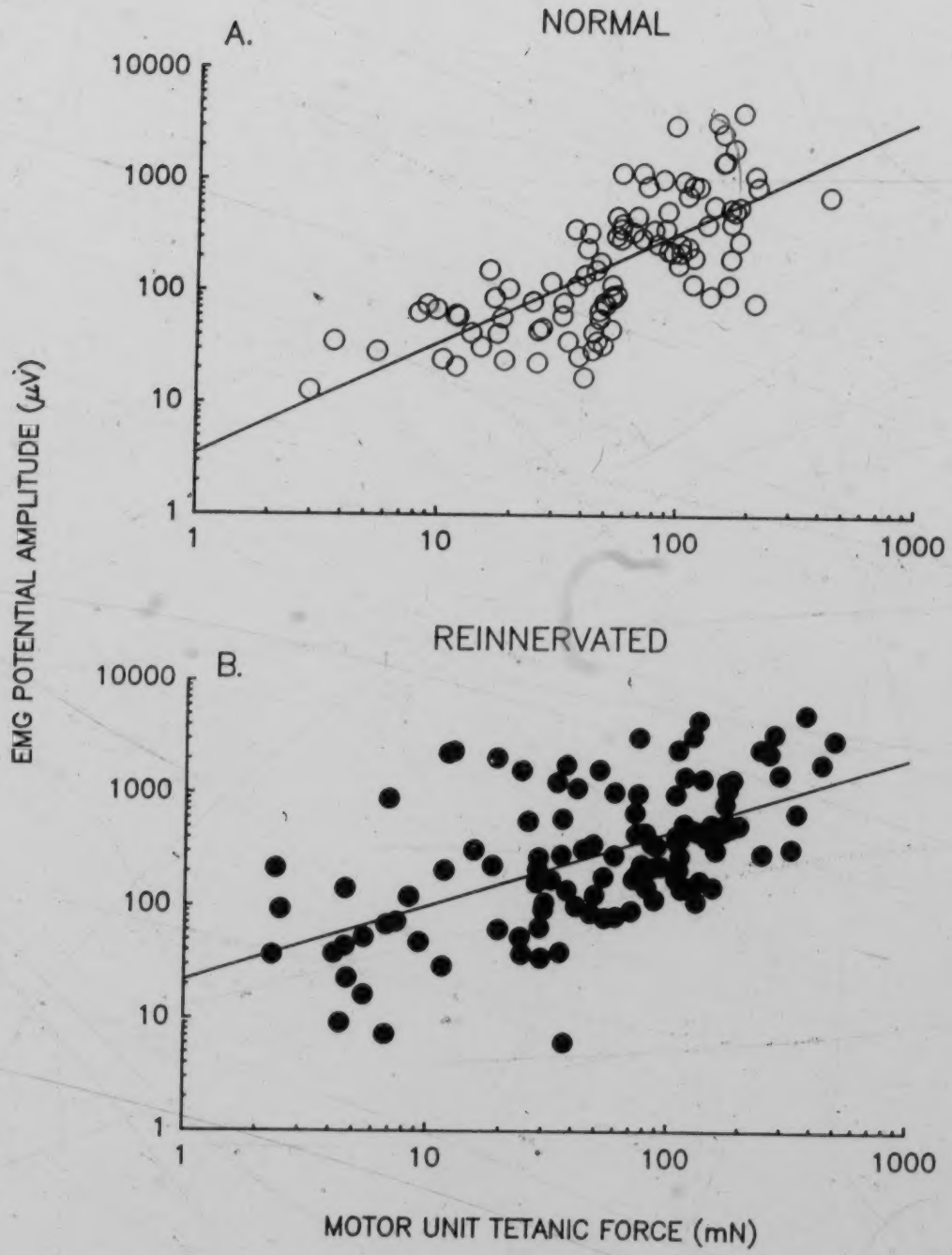
these data suggest that the electrical activity associated with motor unit activation increases as an approximate square root function of its force, which is consistent with the findings of Milner-Brown and Stein (1975). However the correlation coefficients are low in each case (0.53 to 0.59) indicating that factors other than the size (force) of the motor unit are contributing to the variation in EMG. In spite of this high variability in the data, it can be said that unit EMG amplitude increases similarly with twitch force in normal and reinnervated muscle and the altered distribution of muscle unit fibres in the latter appears to have no effect.

When maximum tetanic force is plotted against EMG amplitude the slope is different between normal and reinnervated units (Figure 4.6). Since no differences were found between intramuscular and surface recordings, the data from both have been pooled. EMG varies linearly with unit tetanic force in normal units while in reinnervated units the slope is closer to the square root relationship seen with twitch force. However, it is clear that the degree of scatter in the data from reinnervated muscle is greater resulting in a much poorer correlation between force and EMG.

### 4.III.3 Twitch to Tetanus Ratios

The result that the twitch force-EMG relation is the same in normal and reinnervated muscles while the tetanic force-EMG relation is not, was thought to

Figure 4.6 Relation between tetanic force and EMG amplitude in normal and reinnervated motor units. In A the data from normal units show that EMG amplitude increases linearly with tetanic force ($m=0.98 \pm 0.10$; $r=0.72$; $n=99$) whereas in reinnervated units it is closer to the square root relation seen with twitch force ($m=0.66 \pm 0.09$; $r=0.58$; $n=113$).


A.
NORMAL
10000
1000
100
10
1
1
10
100
1000
B.
REINNERVATED
10000
1000
100
10
1
1
10
100
1000
EMG POTENTIAL AMPLITUDE (μV)
MOTOR UNIT TETANIC FORCE (mN)

be due to differences in mechanical rather than electrical properties between the two muscle groups. Twitch force is plotted against tetanic force in Figure 4.7 and the different slopes for normal and reinnervated muscle confirms this hypothesis indicating that the small force producing units in normal muscle tend to potentiate more than the larger ones. In contrast, in reinnervated muscle all units potentiate to approximately the same extent regardless of their size.

### 4.III.4 Glycogen Depleted Units

#### 4.III.4(a) Force and EMG in Depleted Units

A representative sample of normal and reinnervated units were glycogen depleted (see methods in chapter 2) and the number, size and spatial location of all the constituent muscle fibres were directly determined. The cumulative frequency distributions in Figure 4.8 show that the range and distribution of EMG amplitudes from the total unit population is well represented by the depleted units. The Kolmogorov-Smirnov test showed no significant difference between the frequency distributions from the whole population of sampled units and that from the depleted units in either the normal or reinnervated group.

Figure 4.7 Comparison of the twitch-tetanus relation in normal and reinnervated motor units. Twitch force is plotted against tetanic force and shows that the small units in normal muscle (A) potentiate more than the larger ones ($m=0.64 \pm 0.04$; $r=0.85$) while in reinnervated muscle (B) all units potentiate to approximately the same degree ($m=0.89 \pm 0.04$; $r=0.89$). The slopes of the two lines are significantly different ($P < 0.05$).

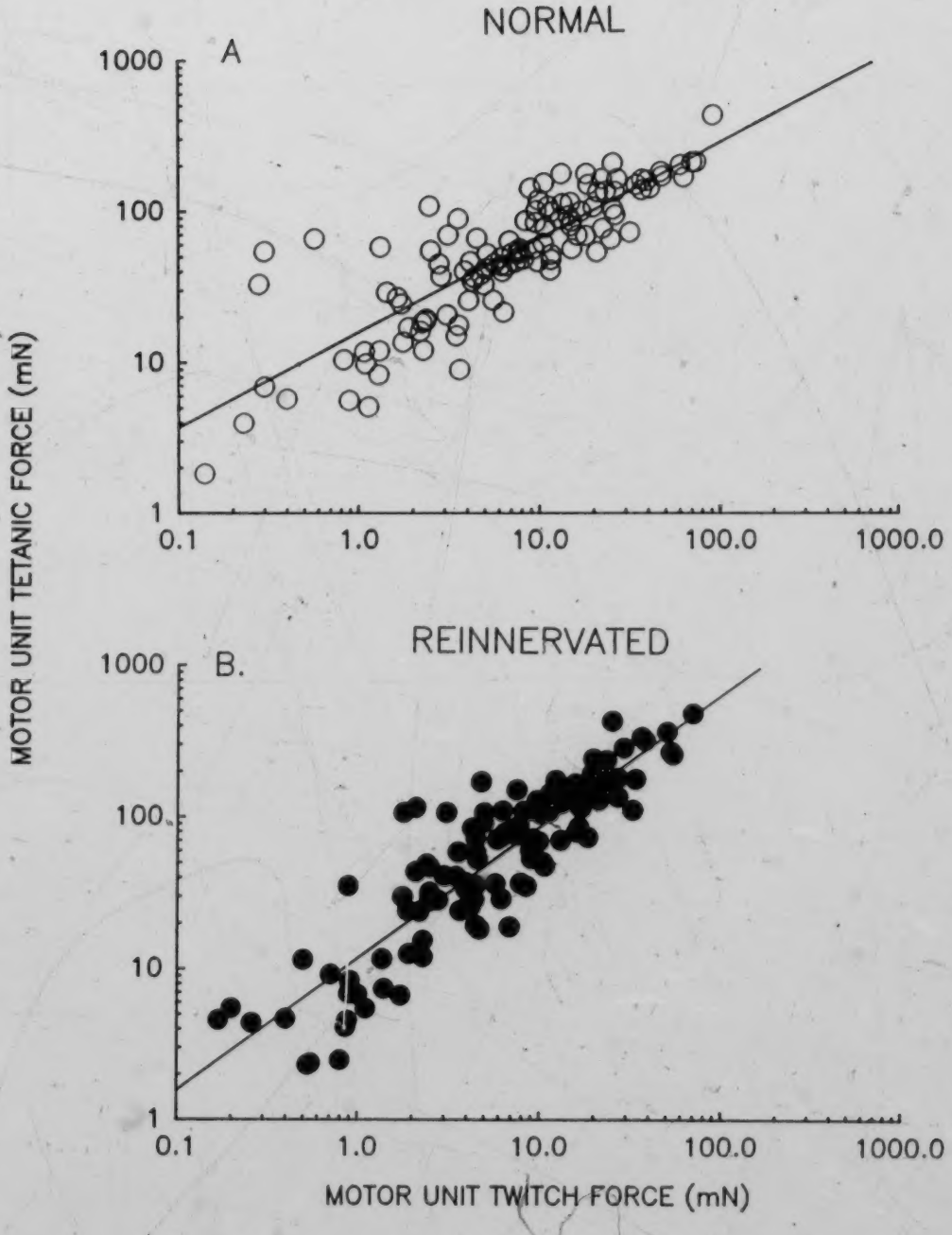
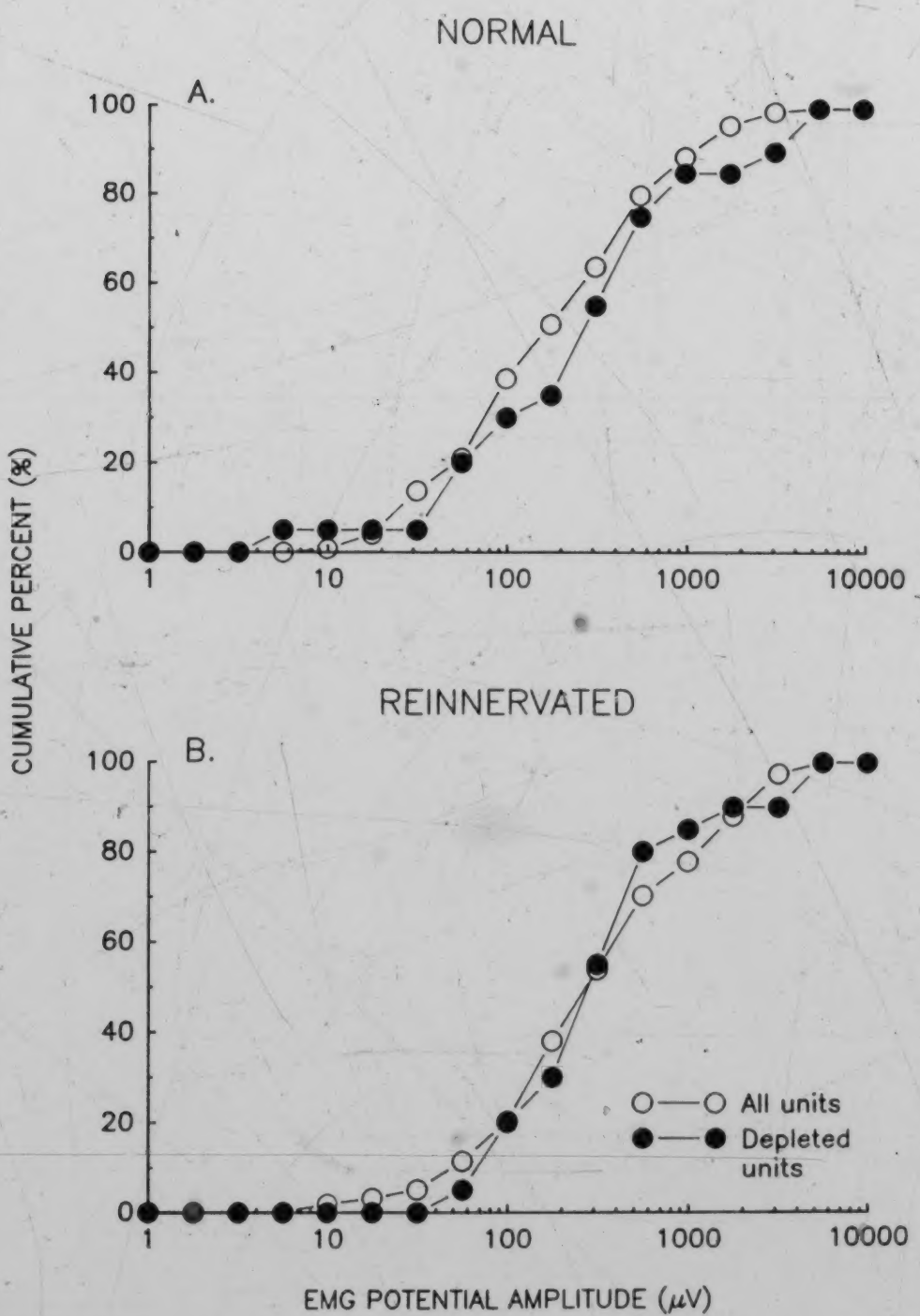

NORMAL
A
1000
100
10
1
0.1
1.0
10.0
100.0
1000.0
REINNERVATED
B.
1000
100
10
1
0.1
1.0
10.0
100.0
1000.0
MOTOR UNIT TETANIC FORCE (mN)
MOTOR UNIT TWITCH FORCE (mN)

Figure 4.8 Cumulative frequency distributions comparing the EMG amplitudes from glycogen depleted units to those from the whole population. These cumulative distributions are not significantly different ($P > 0.05$, Kolmogorov-Smirnov test) in either normal or reinnervated muscle between the whole population and the glycogen depleted population of units.

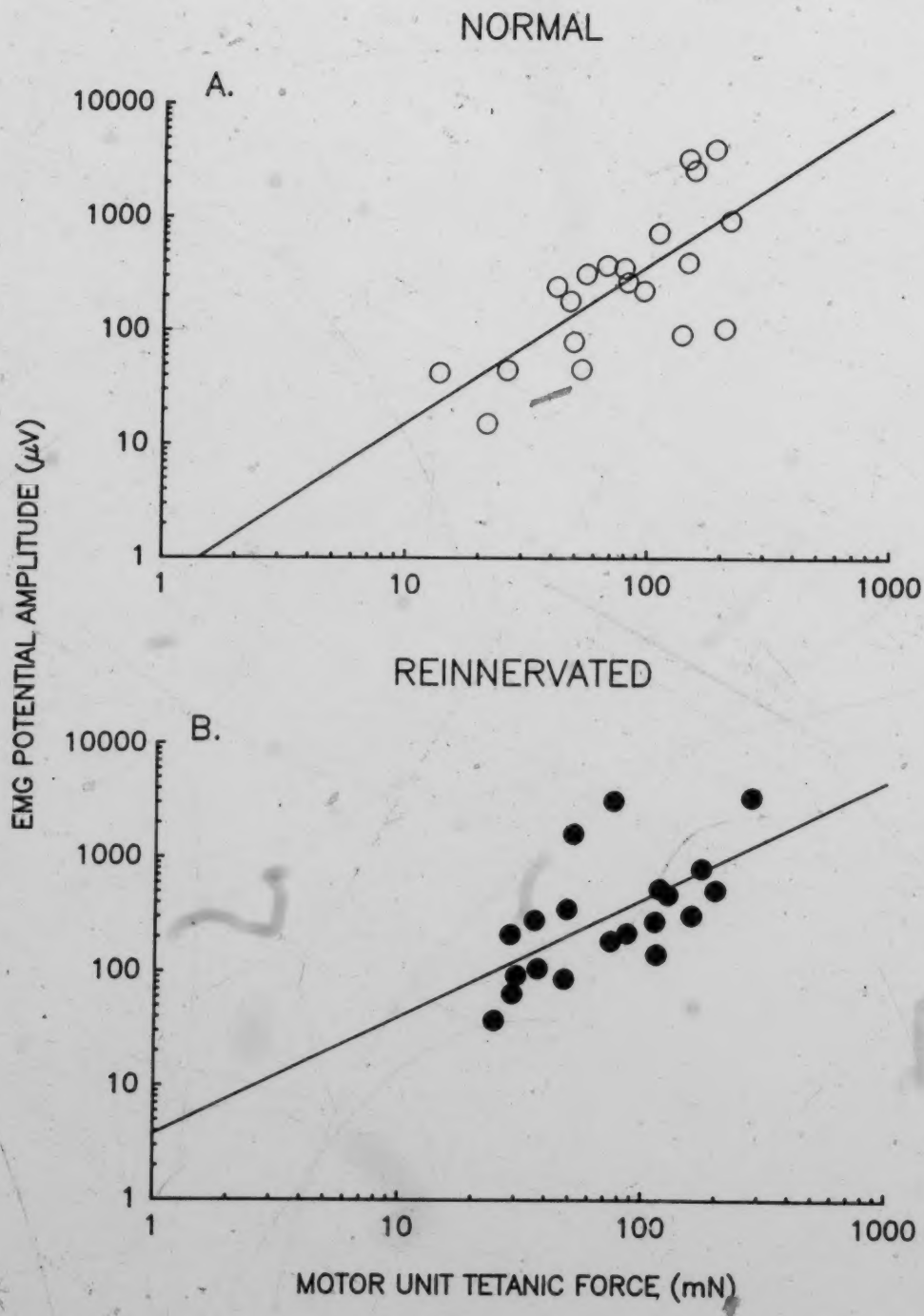

NORMAL
A.
100
80
60
40
20
0
1
10
100
1000
10000
CUMULATIVE PERCENT (%)
REINNERVATED
B.
All units
Depleted units
EMG POTENTIAL AMPLITUDE (μV)

In both normal and reinnervated depleted units tetanic force varies approximately linearly with EMG amplitude (Figure 4.9) and also with EMG area (not shown). When the whole population of reinnervated units was considered, as in Figure 4.6B, EMG varied with approximately the square root of tetanic force yet for the depleted units this relation is linear. Due to the large scatter in the data from the whole population of sampled units seen in Figure 4.6, it is perhaps not surprising that the smaller sample of depleted units shows a different relation between force and EMG. In fact, it is somewhat instructive in that it draws attention to the variability inherent in this highly complex system. It also indicates that ultimately, EMG is a poor predictor of force.

### 4.III.4(b) EMG and Muscle Fibre Distribution

Thus far, the results have shown that the altered spatial distribution of muscle unit fibres in reinnervated muscles results in a larger EMG signal for a given force but that the way in which this signal varies with force remains the same. When the spatial location of each glycogen depleted unit was examined with respect to force and EMG it was found that normal units with the smallest EMG tended to be located in the deep compartment and the largest ones were more superficial (see Chapter 5). These deeper lying units also tended to produce smaller forces and contain smaller muscle fibres thus the smaller EMG signals from the deep lying units are likely due to factors associated with unit size rather than to any signal decrement with depth.

Figure 4.9 Relation between tetanic force and EMG amplitude in the glycogen depleted motor units. The slopes of the lines are 1.39 ± 0.32 (r=0.72) and 1.15 ± 0.29 (r=0.68) for normal (A) and reinnervated (B), respectively.

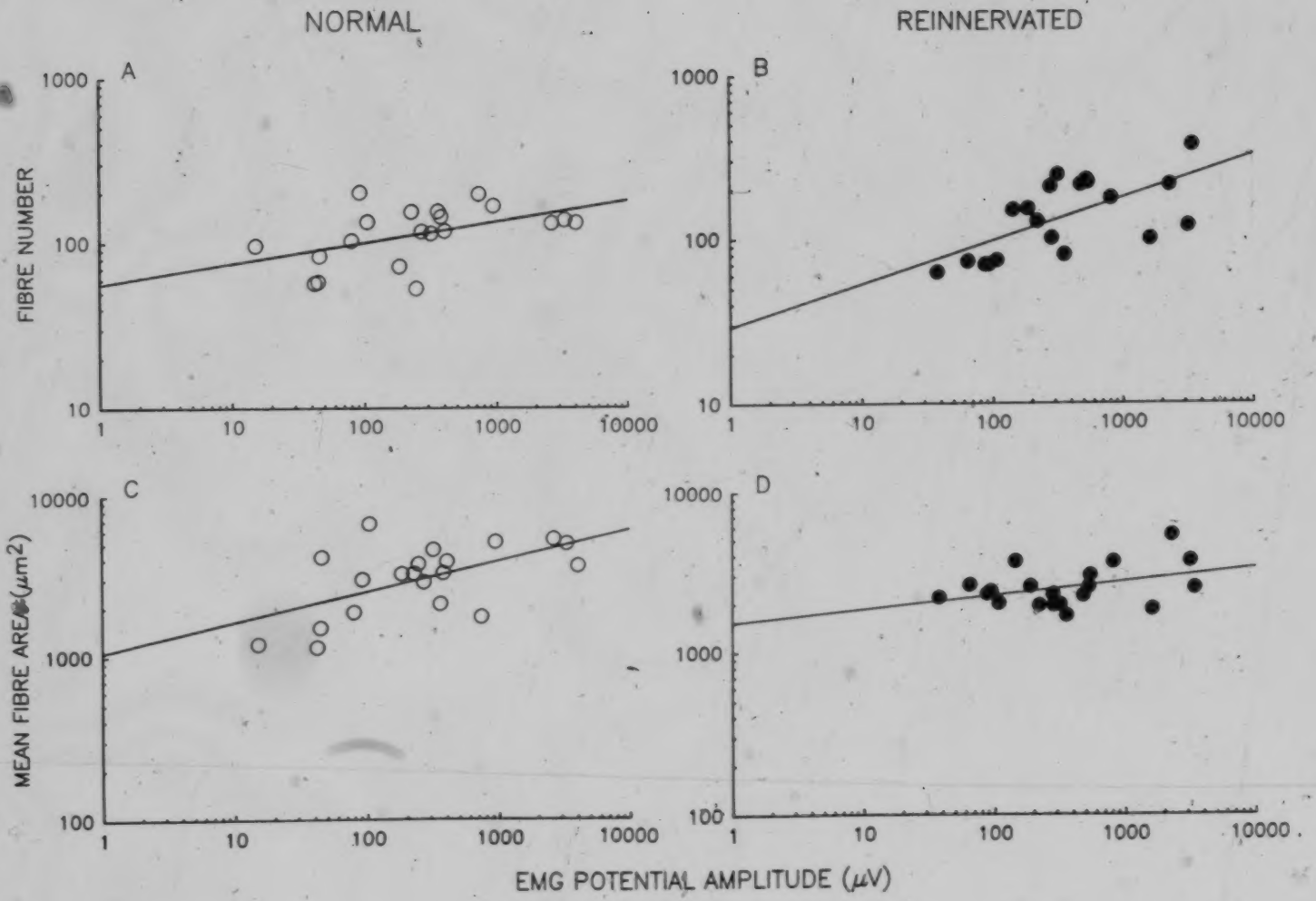

NORMAL
A.
10000
1000
100
10
1
1
10
100
1000
REINNERVATED
B.
10000
1000
100
10
1
1
10
100
1000
EMG POTENTIAL AMPLITUDE (μV)
MOTOR UNIT TETANIC FORCE (mN)

In contrast, in reinnervated muscle both small and large force producing units could be found in any region of the muscle. In spite of these geometric differences between normal and reinnervated muscle units, the relation between twitch force and EMG amplitude remains the same. Thus neither the altered spatial distribution (ie, clumped fibres) nor the altered regionalization of reinnervated muscle units seem to affect the EMG-force relation. The observed variations in EMG with force must then be due to factors other than the distribution or spatial location of the muscle unit fibres.

### 4.III.4(c) EMG and Muscle Fibre Number or Area

Having ruled out that spatial factors of muscle unit fibres contribute to the variation in the EMG, other factors likely to influence the magnitude of the recorded electrical signal are the number and size of constituent muscle unit fibres. From the direct fibre counts for each individual motor unit, which are plotted against EMG amplitude in Figure 4.10, it is evident that an increase in fibre number accounts for very little of the increase in EMG amplitude in normal muscle. Although there is a greater tendency for reinnervated units with larger EMG values to have a greater number of fibres here too fibre number accounts for relatively little of the increase in EMG. Considering mean unit fibre areas, in normal units this also contributes very little to the variation in recorded EMG and in reinnervated units the variation in mean unit fibre area is so small

Figure 4.10 Relation between EMG amplitude and fibre number and area in normal and reinnervated motor units. The slope of the relation between EMG amplitude and fibre number is less steep with normal units (A; m= 0.13 ± 0.05; r=0.48) than with reinnervated units (B; m= 0.26 ± 0.07; r=0.64) while the opposite is true for the relation between EMG amplitude and mean fibre area. In this case the slopes are 0.19 ± 0.06 (r=0.57) for normal (C) and 0.09 ± 0.05 (r=0.39) for reinnervated (D). The sum of the slopes from A (fibre number) and C (mean fibre area), is 0.33 which represents an estimate of the contribution of total cross-sectional area to EMG amplitude.


NORMAL
REINNERVATED
A
B
C
D
FIBRE NUMBER
MEAN FIBRE AREA (μm²)
EMG POTENTIAL AMPLITUDE (μV)
1000
100
10
10000
1
10
100
1000
10000

compared to that of EMG amplitude that the slope of the regression line is not significantly different from zero.

The product of fibre number and mean area from single units gives the total unit cross-sectional area which was previously shown to account for nearly all of the variation in force production in both normal and reinnervated units (Chapter 2). By comparison, total unit area accounts for only about 30% of the variation in EMG amplitude or area. Thus the number and area of muscle unit fibres contribute much less to the variation in the motor unit EMG signal than was expected.

## 4.IV DISCUSSION

### 4.IV.1 EMG and Muscle Fibre Geometry

We have found that with our recording conditions the EMG signal from single active motor units in normal muscles, recorded either intramuscularly or from the surface, varies approximately as the square root of twitch force but linearly with tetanic force. The same relations are seen using either the peak to peak amplitude or the integrated area as the parameter measure of EMG. As mentioned previously, Milner-Brown and Stein (1975) found a similar square root relation between surface EMG amplitude and twitch force in human first dorsal interosseus muscle. A somewhat different result was reported by Clamann and Robinson (1985) who found a parabolic relationship between integrated intramuscular EMG and tetanic force in cat medial gastrocnemius motor units. Large motor units produced relatively more electrical activity than small units. Finally, Lippold (1952) found a linear relation between integrated surface EMG and tetanic force in human gastrocnemius muscle. In this study we have found a square root relation between EMG and twitch force and an approximately linear relation between EMG and tetanic force in normal muscle. Further, the number and size of active muscle fibres had little influence on the magnitude of

the signal.

Theoretically, if muscle unit fibre number were to remain constant, and differences in force output were due entirely to differences in fibre size, maximum force output of a motor unit should increase with the square of muscle fibre diameter (area). If EMG can be expected to vary directly with fibre diameter (Rosenfalck, 1969), from this line of reasoning it follows that under these conditions EMG amplitude should increase as the square root of force. At the other extreme, with fibre area constant and variations in force due to differences in the number of fibres, force output should increase directly with fibre number as should the EMG. In this case then, EMG would be expected to vary linearly with force. Since both fibre number and area vary considerably between units (see chapter 2), the true relation between force and EMG will depend on both and will likely lie somewhere in between a square root and a linear dependence. Ultimately, due to the high variability consistently observed in these data, EMG did not vary predictably with force.

What is contributing to this high degree of variability in the recorded EMG? Some of the possible explanations include variations in 1) the location of active fibres within the muscle, 2) fibre size and conduction velocity, 3) the spatial location of endplates between the electrode recording sites, 4) synaptic latency, and 5) the length and diameter of nerve terminal arborizations. Factors 2) to 5) probably lead to asynchronous spike potentials within units.

### 4.IV.2 EMG and Muscle Unit Spatial Location

The location of unit fibres within the muscle (ie, their distance from the recording electrode) has been suggested by many investigators to play an important role in determining EMG signal amplitude such that deep and superficial units would be unequally picked up at the surface (Lippold, 1952; Buchthal, 1973). While this argument seems obvious, the importance of location was not borne out by our experimental observations on the rat TA. This is likely due to the small size of the muscle and the relatively large muscle unit territories which can cover up to 30% of the muscle cross-sectional area (see Chapter 5). In large bulky muscles location of the unit may become more important. Using a different line of reasoning, from elementary physics we know that for a point source of current, the potential declines with distance from the source. Since muscle fibres are not point sources of current and a large number of widely distributed fibres are conducting at once (probably asynchronously) during activation of a single motor unit, the exact relation between signal amplitude decrement and distance from the recording electrode is difficult to clarify. Furthermore, the resultant wave from the activity of all fibres of a motor unit may involve some degree of cancellation due to asynchrony in the spike potentials. If we could superimpose the individual action potentials from all muscle fibres in a unit it would be expected that some portions of these waves would be in

opposite phases.

A recent study by English and colleagues (1989) where EMG was measured with wire electrodes within different muscle compartments has shown that the signal from a large force producing unit recorded in an adjacent compartment can be larger than that from a small unit within the same compartment. This indicates a much smaller decrement of the signal than has been predicted from calculations of tissue filter functions (Lindstrom, 1970) where small displacements of a needle electrode in the order of 100 $\mu$m cause about a 75% decrease in the peak to peak amplitude. Clearly, the signal decrement must somehow be a function of distance between the current source and the recording site but determination of the exact relationship is complicated by the architecture of muscle fibres and the different electrode designs used by different investigators.

In view of the many possible sources of variability contributing to the summed EMG potential, it is perhaps not so surprising that total unit cross-sectional area (the product of fibre number and area) accounts for only about 33% of the differences in EMG between units (Figure 4.10). Thus, the contribution of total unit area to EMG amplitude is much lower than that obtained for force output (88%) in Chapter 2. The additive effects of all of the other factors listed above may account for the rest and may explain the large degree of scatter in the EMG-force relation.

Muscle unit fibre density seems to be a much more important factor determining the magnitude of EMG than fibre number, size or location in view of the significantly higher values obtained from the highly clumped reinnervated units (Figure 4.3). In addition to the increased current density produced by this clumped arrangement of muscle fibres, the terminal arborizations in reinnervated motor units are likely to be shorter. This could effectively reduce asynchrony in the onset of individual action potentials leading to a greater summated EMG.

### 4.IV.3 Twitch-Tetanus Ratio

An interesting result from this study is the difference in the twitch to tetanus ratio in normal and reinnervated muscle. Normally this varies with unit size in the rat TA such that the relative increase in force from twitch to tetanus becomes less as units get larger (Figure 4.7). In reinnervated muscle there was no change in the ratio with size; the same proportional increase was found for all units. This suggests that the nature of the activation process is somehow different after reinnervation. The amount of force developed in response to a single stimulus depends on spatial and temporal aspects of calcium availability as well as on the relative sensitivity of the contractile elements to calcium. Slow twitch muscle fibres have half the volume fraction of t-tubule and terminal cisternae membrane as well as half the volume density of junctional feet than

fast twitch fibres (Eisenberg, 1985; Franzini-Armstrong, 1988) suggesting that both the penetration of excitation and its coupling to calcium release are of smaller magnitude. These anatomical differences could lead to slow fibres being less fully activated during a single twitch than the fast fibres, resulting in a smaller twitch to tetanus ratio.

Considering now the reinnervated motor units, many structural changes are known to occur in muscle fibres following denervation. The t-tubules and terminal cisternae have been shown to lose their usual transverse orientation to one another within 2 weeks of denervation (Gauthier and Dunn, 1973). In addition to the commonly observed denervation atrophy (Engel et al., 1966) and the regression towards a less differentiated state (Carraro et al., 1985), the normal alignment of the myofibrils is also disrupted after denervation (Gauthier and Dunn, 1973). It has been estimated that the rate of nerve regeneration following transection injury is about 1 mm per day, corresponding to the slow rate of axonal transport (Sunderland, 1978; Lasek, 1986). Taking into account the delay before axons begin to regrow after the initial injury, the time required for sprouts from the sectioned nerve to grow through the scar tissue and the distance (about 10 mm) over which the sprouts must travel to reach the TA muscle in our experimental animals, there is sufficient time for these degenerative changes to occur before nerve-muscle contact can reverse them. Thus the degree of reorganization of muscle fibre structure and properties that

must take place during reinnervation is considerable. If the suggestion put forward by Westgaard and Lomo (1988) that each fibre type has an 'adaptive range' over which its properties may be adjusted is correct, then one would expect motor units in reinnervated muscle to become more similar in their properties since they contain a mixture of fibres formerly of several different histochemical types which will have different adaptive ranges. This type of organization could lead to the similar twitch to tetanus ratios found among the reinnervated unit population.

In summary, the major findings of this present study are that the spatial location of a motor unit within the rat TA muscle has no influence on the magnitude of the EMG signal but that increased muscle fibre density in reinnervated motor units leads to an enhancement of the EMG signal. Differences in muscle unit fibre number (innervation ratio) and fibre area in both normal and reinnervated motor units contribute relatively little (about 33%) to the variation in EMG signal magnitude which is due to an alteration of the EMG signal by other factors as discussed above. EMG varies positively with twitch and tetanic force in both normal and reinnervated units but the variability in EMG was large. The twitch to tetanus ratio in normal motor units increases with unit size while it is essentially the same for all reinnervated units. It is proposed that the reinnervating nerve is unable to respecify muscle properties associated with activation beyond a certain limit and the normally diverse population of

motor units becomes more homogeneous.

# CHAPTER 5

# SPATIAL ANALYSIS OF MOTOR UNIT TERRITORIES

## 5.I INTRODUCTION

The spatial organization and the size or density of muscle unit fibres and the reorganization after injury or disease has been estimated electromyographically in a number of different human muscles (Buchthal et al., 1957; Erminio et al., 1959; Stalberg and Thiele, 1975; McComas et al., 1971). Results from these studies indicate a diffuse distribution of normal unit fibres and dense clusters of reinnervated or diseased fibres. The glycogen depletion technique of Edstrom and Kugelberg (1968) provided a more direct method of examining the spatial distribution of muscle unit fibres. In this study muscle unit territorial size was expressed as a percentage of the whole muscle cross-section and the mean value from 13 rat TA units was 17%. Using the same approach values of 12% and 15% have been reported from rat (Brandstater and Lambert, 1973) and cat (Bodine et al., 1987), respectively.

From a statistical analysis of fibre adjacencies within defined regions of the muscle units Brandstater and Lambert (1973) found that the fibre distribution was not different from random while Willison (1980) found fewer adjacencies than

would be expected from a random distribution, indicative of a process of segregation or active spreading. More recently Bodine et al., (1988) analysed fibre distributions in the cat TA muscle using a number of tests, including adjacencies, nearest neighbour and interfibre distances and found a tendency for randomness in some units but not in others. Their general conclusion was that there is a greater tendency for grouping than segregation suggesting that some mechanism is restricting the upper limit of territory size.

The distributions of muscle unit fibres in reinnervated muscle have not been analyzed statistically yet the high degree of clumping indicates that the distribution is neither random nor segregated. The obvious differences in spatial patterns between normal and reinnervated muscle units indicates that the pattern of axonal branching must be different during development compared to that during regeneration. Although it is not possible to directly determine how the axonal branching patterns of individual motor units come about, examination of the distribution of the muscle fibres they innervate may provide some interesting clues. In this study we have examined the spatial distributions and territorial size of rat tibialis anterior (TA) muscle fibres from normal and reinnervated glycogen depleted motor units in order to clarify the relationship between territory size and fibre number. The distributions of muscle units can provide indirect information on the possible role of intramuscular endoneurial tubes in the branching of regenerating motor nerves. English and Weeks (1984) have demonstrated in the

cat lateral gastrocnemius that discrete compartments exist which are innervated by different primary branches from the parent nerve as it enters the muscle. These authors also showed that single motor unit territories are confined within the boundaries of a compartment indicating that all branches of individual motoneurones occur distal to the primary branch. Evidence has also been obtained for compartmentalization in the cat tibialis anterior (Iliya and Dum, 1984) and the rat extensor digitorum longus (Balice-Gordon and Thompson, 1988) muscles. In the present study we also examined whether similar discrete compartments exist within the rat TA and if so, whether they are re-established following reinnervation. This would reflect the ability of regenerating axons to recognize and follow the empty endoneurial tubes left behind at a major branch point.

## 5.II METHODS

### 5.II.1 Motor Unit Isolation

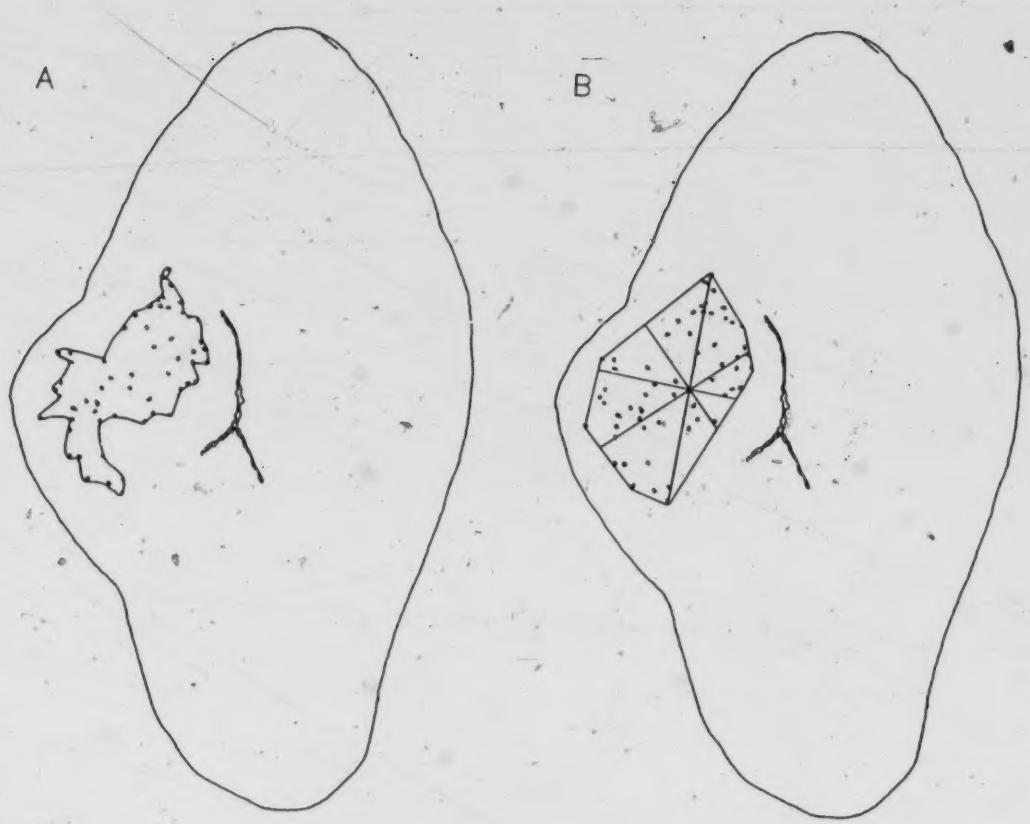
Single motor units were physiologically isolated for glycogen depletion as described in detail previously. Briefly, repetitive stimulation was delivered through fine ventral root filaments supplying the surgically isolated tibialis anterior muscle to deplete muscle units of their stored glycogen. Muscles were immediately removed upon cessation of stimulation, cut into three blocks and frozen in isopentane cooled in liquid nitrogen. Successive serial cross-sections (10 $\mu$m) were stained for glycogen with periodic acid-Schiff (PAS) (Pearse, 1960) to visualize the muscle unit and for myosin ATPase following acid- (Brooke and Kaiser, 1970) and alkaline-preincubation (Guth and Samaha, 1970) for determination of fibre type. Camera lucida drawings were made from the PAS-stained cross-sections where the whole muscle cross-section as well as all the glycogen depleted muscle fibres were outlined. As described previously, such drawings were made from a number of sections taken at different proximodistal levels along the muscle. The cross-section yielding the largest number of muscle fibres was selected for the present territorial analysis.

### 5.II.2 Muscle Unit Territories

Motor unit territories were determined from these drawings in two ways as shown in Figure 5.1. The minimum territory was produced by drawing a line from each fibre to its closest outlying neighbour, while the maximum territory was determined by connecting the outermost fibres according to the method described by Edstrom and Larsson (1987). Total muscle cross-sectional areas and the minimum and maximum muscle unit territorial areas were measured by planimetry on a digitizing tablet.

For analysis of muscle unit spatial location muscles were divided into four regions. Examination under low power magnification of the TA muscle nerve revealed that it divides consistently into two and occasionally three primary branches within about 1-2 mm of its point of entry into the muscle. This anatomical feature was taken as supporting evidence for the existence of discrete compartments. After carefully transecting one of these branches to the TA, and denervating all other hindlimb muscles, fatiguing stimulation was delivered via the sciatic nerve in an attempt to glycogen deplete the remaining 'compartment'. Although the muscle appeared somewhat ischemic and overall quite pale on the PAS-stained cross-sections, the entire deep region was identifiably paler than the rest of the muscle. This technique is difficult to perform successfully due to the invasive dissection required to isolate a primary branch of the nerve and the high

Figure 5.1 Camera lucida drawings of a single muscle unit showing the minimum and maximum territorial measurements. In A a line has been drawn from each fibre to its closest outlying neighbour and represents the minimum territory for that unit. In B the lines are drawn between the outermost fibres according to the method of Edstrom and Larsson (1987) and represent the maximum territory for that unit. Note the irregular spacing of the unit fibres about the territorial boundary.


A
B

probability of damaging the blood supply. An observation that lends further support to the existence of a deep and superficial compartment is that the small population of type I or slow oxidative fibres is exclusively found only in the deep region. Thus, each muscle was considered to have two major regions, deep and superficial. From the observations of Edstrom and Kugelberg (1968) that unit territories tend to occupy an average of 17% of the muscle cross-section, the superficial region was divided into three zones from medial to lateral to facilitate description of their spatial localization.

### 5.II.3 Muscle Fibre Areas

Muscle fibre cross-sectional areas were measured from two areas in each of five regions: deep, central, medial, superficial and lateral. Measurements were made by manually outlining the fibre boundaries with a trackball on grey level pictures projected on the video monitor of the Computer Image Processing System described in Chapter 2.

## 5.III RESULTS

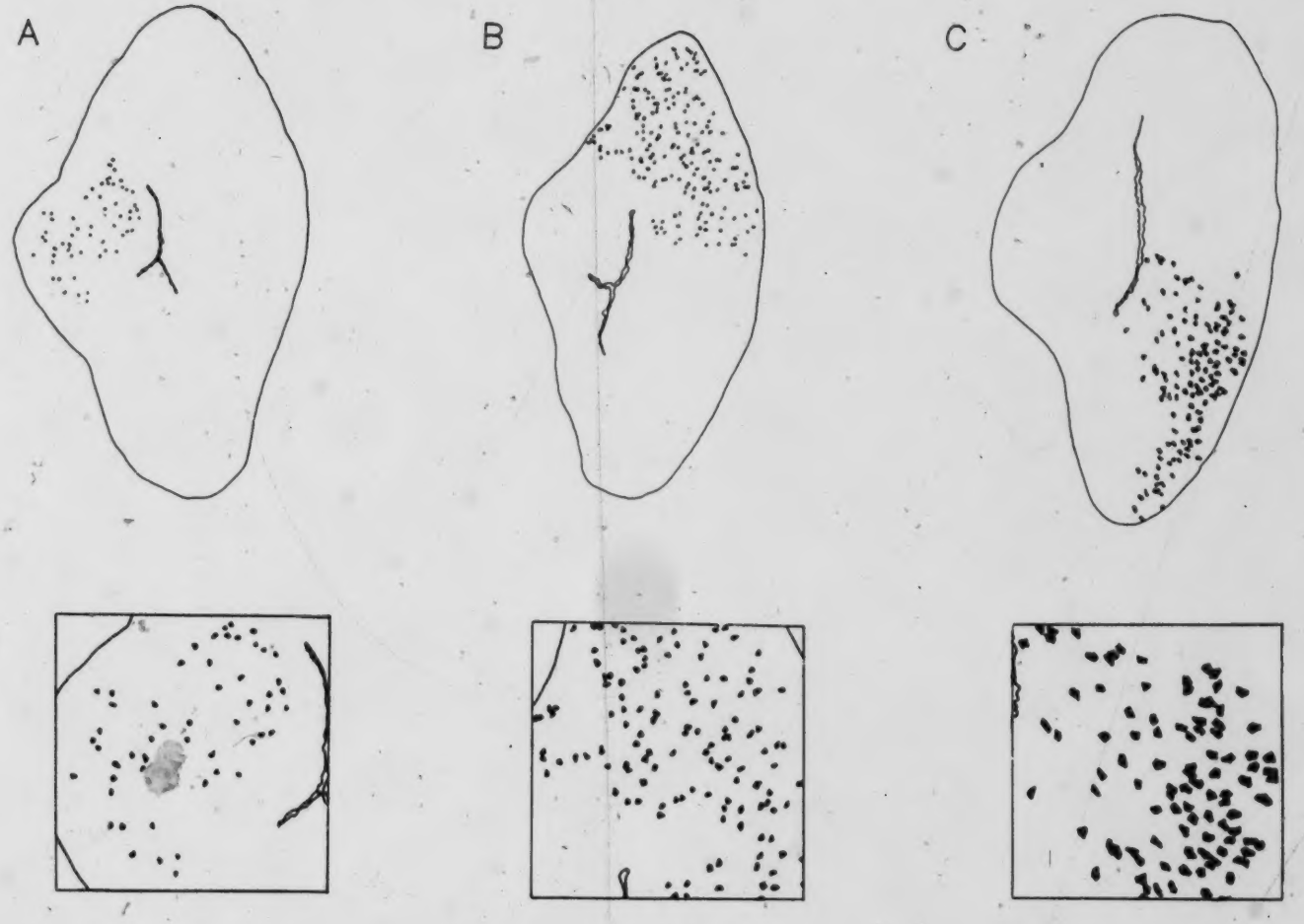
### 5.III.1 Normal Muscle Unit Fibre Distribution

The muscle unit territories from normal motor units were generally confined to one of four regions. Units occupy either the deep, or superficial regions and within the superficial region they may be medially, centrally or laterally located. Eighteen out of the 20 units examined could be said to follow this general pattern and Figure 5.2 shows some representative examples of these distributions. The unit in A is one of six units located in the deep region (2 SO, 2 FOG, 1 Fint and 1 FG). The middle panel (B) shows a medially located unit, of which there were also six (1 FOG, 2 Fint and 3 FG). Four units were found in the lateral region (2 FG and 3 Fint) an example of which is given in the right of the figure (C) and two (Fint and FG) were in the central region. From all the units examined only two deviated from this general regional distribution pattern. One was found to extend across the deep well into the superficial region, while the other spanned nearly the entire superficial region from medial to lateral.

Additionally, the FG and Fint muscle units could be found in all regions, FOG units were found deep and medially, while SO units were only in the deepest part of the muscle. The incidence of different muscle unit types found in each

Figure 5.2 Spatial location of single motor units from normal muscle. These camera lucida drawings are oriented such that the top margin is medial, the bottom is lateral and left and right are deep and superficial, respectively. The deep lying unit in A is type SO and developed a force of 26 mN with a fibre number of 58. In B the unit is Fint, developed a force of 110 mN and contained 197 fibres while in C the unit is type FG and developed a force of 146 mN with 137 fibres. The magnification in each of the drawings across the top of the figure is 14X while the magnified portions of the units (boxes) are 28X. Note that the unit in C has the largest fibre sizes.

NORMAL



region in this study agrees well with the regional occurrence of the particular fibre types in the whole muscle. We observed that type SO fibres, identified by myosin ATPase histochemistry, are normally confined to the deeper regions. Type FOG fibres become increasingly less predominant along a deep to superficial gradient while the reverse is true for type FG and Fint. The relative proportions of each type are given in Chapter 3 and agree well with previous reports of regionalization of fibre types in the rat TA (Pullen, 1977; Armstrong and Phelps, 1984).

Taken together, the observation that the TA muscle nerve divides into two primary branches as it enters the muscle, and the fact that with one exception, units were either in the deep or superficial regions but not both, it would appear that at least two discrete compartments exist in the rat TA and that the motor unit territorial boundaries are within the boundaries of the compartment in which they are located. This further suggests that all branches of individual motoneurones occur distal to the primary branch.

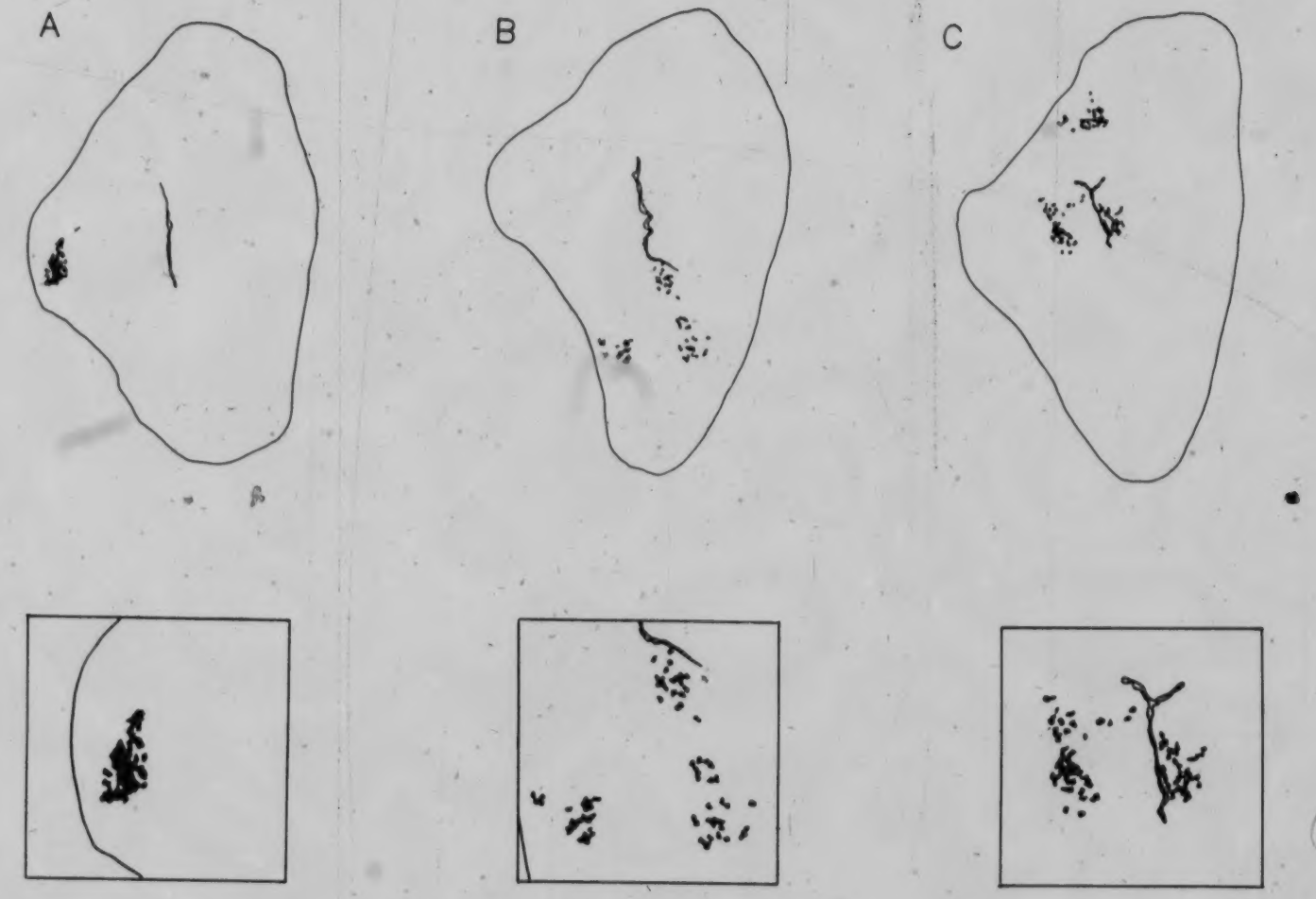
### 5.III.2 Reinnervated Muscle Unit Fibre Distribution

In Chapter 2 a depleted unit from reinnervated muscle was shown (Figure 2.7) which indicated the high degree of clumping that can occur in these muscle units. Clumping has been shown in experimentally reinnervated muscle

(Kugelberg et al., 1970) but a direct territorial analysis of entire muscle units from reinnervated muscle has not been previously reported. In general, most reinnervated units were found in one of the four regions as normal however the muscle fibre distribution patterns were highly variable. In Figure 5.3 examples are given of the three most common distribution patterns. The first example (A) shows a tightly clumped and very small FOG unit located in the deep region. Five units out of 20 exhibited this tight packing of fibres (1 SO, 3 FOG, 1 FG) and four of them were located close to or along the deepest margin of the muscle, close to the motor point. This tight clumping may arise from terminal sprouting of the axon shortly after it enters the muscle. The unit in the middle panel (B) shows a relatively diffuse distribution of muscle fibres. This pattern was observed in two units, both of which were SO. Note that this SO unit is located in the lateral region indicating that the SO fibres in reinnervated muscle are no longer confined to the deep compartment. The most common distribution pattern, exhibited by 12 of the units (1 SO, 4 FOG, 5 Fint, 2 FG), consisted of groups of clumps separated by variable distances with a few singly occurring fibres like that shown by the Fint unit on the far right (C). The most medial clump of fibres of this unit is located close to the region where the nerve enters the muscle. The tendon is drawn here to show that at least one branch was able to grow around this structure. Three units showed this apparent branching pattern around the tendon. It seems unlikely that the relatively large distances between

Figure 5.3 Spatial location of single motor units from reinnervated muscle. These camera lucida drawings are oriented as in the previous figure with the top margin medial, the bottom lateral and left and right deep and superficial, respectively. The deep lying unit in A is type FOG and developed a force of 29 mN with a fibre number of 73. In B the unit is SO, developed a force of 47 mN and contained 70 fibres while in C the unit is type Fint and developed a force of 86 mN with 128 fibres. The magnification in each of the drawings across the top of the figure is 14X while the magnified portions of the units (boxes) are 28X.

REINNERVATED



clumps in this unit could result from terminal sprouting and suggests that some axonal branching must have occurred higher up (ie, more proximally).

One final unit to be considered was strikingly different from the rest in that it consisted of two major clumps. One clump was located deep in the muscle and the other was superficial. During fatiguing stimulation this unit had a very stable unitary EMG and there was no reason to suspect the presence of contamination by another unit. Assuming this is true, this unit provides a striking example of the distances over which motoneurone branches can grow. Alternatively, it is possible that this motoneurone branched proximal to the primary branch and grew into both ramifications as it entered the muscle. The fact that this was the only such observation indicates that the majority of axons are prevented from branching proximally, perhaps from similar cellular mechanisms as occur during development. These arguments will be considered further in the discussion.

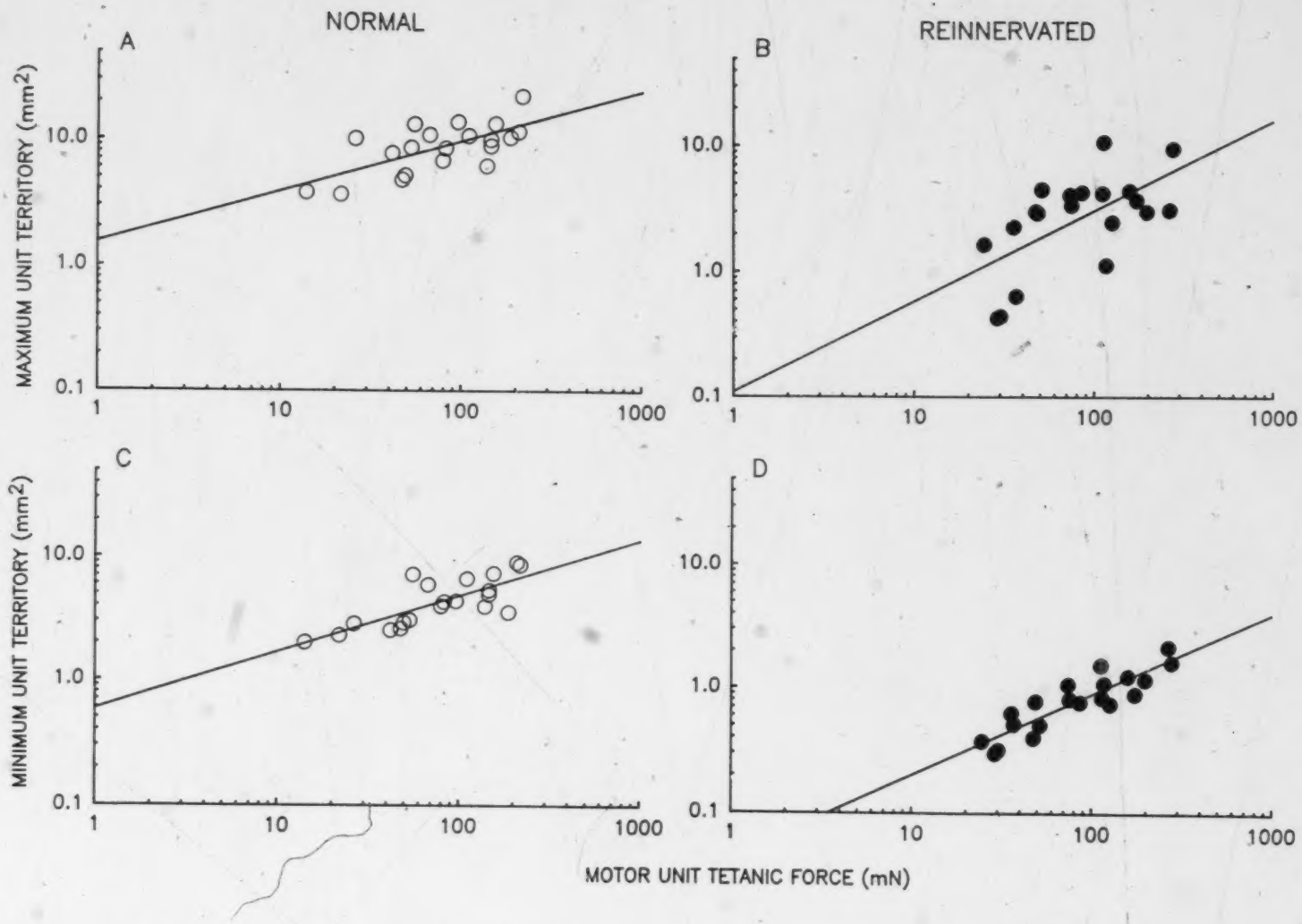
Thus it is evident that the distribution pattern of unit fibres in reinnervated muscle is quite variable ranging from one extreme of densely packed cells to the other extreme of relatively diffuse small clusters. No particular pattern or location was favoured by any of the muscle unit types indicating a loss of the distinct fibre specific regionalization observed in normal muscle. In spite of the diversity in spatial paterning, most units were localized as in normal muscle either in the deep or superficial regions indicating that compartmentalization is preserved.

Examination of the normal and reinnervated units shown in Figures 5.2 and 5.3 provides a visual example of the different contributions of muscle fibre number and size to force development described in Chapter 2. The two units in part C of each figure have similar fibre numbers, 137 and 128, yet the force developed is 146 and 86 mN for the normal and reinnervated units, respectively. Muscle fibre size is clearly larger in the normal unit with a mean value ± standard error of 5018.1 ± 97.7 $\mu m^2$ compared to 1961.7 ± 77.4 $\mu m^2$ in the reinnervated unit.

### 5.III.3 Territory Size and Muscle Unit Force

The observations that motor unit force correlates well with axon diameter in both normal and reinnervated muscle (Gordon and Stein, 1982; Gordon et al., 1986) and that force varies directly with muscle fibre number (see Chapter 2) suggests that the extent of motoneurone branching is size dependent. If the degree of branching (ie, the number of branches) is dependent on the size of the motoneurone, is the extent of its branching (ie, distance over which it branches) similarly related to size? In other words, does the muscle unit territory increase predictably with unit force? Figure 5.4 shows that maximum territory size, reflecting the outermost boundaries of the muscle unit, varies positively with tetanic force in normal and reinnervated muscle but there is more scatter in the data indicating the variability in fibre distribution between units of comparable

Figure 5.4 Relation between muscle unit territory size and tetanic force. In A (normal) and B (reinnervated) motor unit tetanic force is plotted against maximum territory size and in C and D against minimum territory size. In both muscles force varies positively with territory size ($m=0.40 \pm 0.10$; $r=0.68$ for normal (A) and $m=0.74 \pm 0.21$; $r=0.62$, for reinnervated (B)). The points above and below the line in B (scattered and clumped territories, respectively) fit the line quite closely in D. The parallel shift in the lines from maximum to minimum territories indicate an irregular spacing of outer fibres in both normal and reinnervated muscle. The slopes of the regression lines are $0.46 \pm 0.09$ and $0.65 \pm 0.08$ for C and D, respectively and the correlation coefficients increased to 0.78 and 0.89.


NORMAL
REINNERVATED
A
B
C
D
MAXIMUM UNIT TERRITORY (mm2)
MINIMUM UNIT TERRITORY (mm2)
10.0
1.0
0.1
1
10
100
1000
MOTOR UNIT TETANIC FORCE (mN)

force and the slope of this relationship is steeper than in normal muscle. In spite of the high variability in reinnervated muscle fibre distribution patterns the average maximum territory size is significantly smaller than normal with mean values of 9.7 ± 0.1 $mm^2$ and 3.6 ± 0.6 $mm^2$ for normal and reinnervated, respectively. In previous studies territorial size has been expressed in terms of the percentage of the whole muscle cross-section and the mean value from rat (Edstrom and Kugelberg, 1968) and cat (Bodine et al., 1987) TA are 17% and 15%, respectively. By comparison, the mean values from the present study are 21 ± 2% in normal (n=20) and 9 ± 2% in reinnervated muscle (n=20).

When the minumum territory size is plotted against tetanic force as in Figure 5.4 C and D the relationship is similar to that seen when the maximum measure was used but there is a parallel shift in both curves. As described in the methods, the minimum territories were obtained by drawing a line from each fibre to its closest outlying neighbour. This procedure results in the exclusion of areas where no fibres occur and if unit fibres are irregularly spaced the difference between the minimum and maximum values will be large. This difference was large enough to produce an obvious shift in both lines indicating that the spacing of fibres is irregular in both normal and reinnervated units, at least along the territorial boundaries.

### 5.III.4 Fibre Densities

We reported previously that the force capacity of a motor unit varies with the number of muscle fibres it innervates (Chapter 2) and the present results show that force also varies with unit territory size. Since maximum territory size is significantly smaller in reinnervated units the density of fibres (number of fibres per $mm^2$ of territorial area) must be considerably higher. This is indeed the case as shown in Table 6.1. Normal muscle units contain from 6 to 32 fibres per $mm^2$, with a mean value ± standard error of 14.6 ± 1.5, while reinnervated unit fibre density ranges from 14 to 194 fibres per $mm^2$ with a mean of 66.0 ± 11.4. The wide range in fibre density within the maximum territorial areas provides a more quantitative description of the variability in fibre distribution previously described for reinnervated muscle units.

The data from individual units in Table 6.1 indicate their occurrence in either the deep or superficial region of the muscle. In normal muscle, although the sample is small, the deeper lying units tend to have smaller forces, smaller territories and higher fibre densities on average than those in the superficial region. This raises the question whether the pattern of axonal branching is different in the two compartments. Clearly, further experiments are needed to answer this question. The deep lying units in reinnervated muscle also tend to produce smaller forces and have smaller territories than those in the superficial

Table 5.1 MOTOR UNIT TETANIC FORCE, TERRITORY SIZE AND FIBRE DENSITY IN NORMAL MUSCLE.

A. NORMAL

| | TYPE | TETANIC FORCE (mN) | MAXIMUM TERRITORY ($mm^2$) | FIBRE DENSITY (cells/$mm^2$) |
|---|---|---|---|---|
| DEEP | SO | 14.0 | 3.81 | 14.97 |
| | SO | 26.1 | 10.21 | 5.68 |
| | FOG | 21.7 | 3.68 | 26.12 |
| | Fint | 49.2 | 5.22 | 19.73 |
| | Fint | 138.7 | 6.27 | 32.23 |
| | FG | 47.3 | 4.83 | 14.92 |
| | mean | 49.5 | 5.67 | 18.94 |
| | SD | 41.9 | 2.21 | 8.53 |
| | SE | 17.1 | 0.90 | 3.47 |
| SUPERFICIAL | FOG | 82.2 | 8.64 | 13.54 |
| | Fint | 53.0 | 8.70 | 9.54 |
| | Fint | 79.2 | 6.88 | 22.53 |
| | Fint | 110.1 | 10.87 | 18.10 |
| | Fint | 207.7 | 11.75 | 11.41 |
| | FG | 41.6 | 7.83 | 6.77 |
| | FG | 55.2 | 13.37 | 8.53 |
| | FG | 67.0 | 11.02 | 13.07 |
| | FG | 95.9 | 14.01 | 10.99 |
| | FG | 145.9 | 10.16 | 13.49 |
| | FG | 146.0 | 9.04 | 13.05 |
| | FG | 154.5 | 13.61 | 9.62 |
| | FG | 187.1 | 10.57 | 12.49 |
| | FG | 217.2 | 22.60 | 7.39 |
| | mean | 117.3 | 11.36 | 12.18 |
| | SD | 57.0 | 3.76 | 4.04 |
| | SE | 16.5 | 1.08 | 0.88 |

Table 5.1 MOTOR UNIT TETANIC FORCE, TERRITORY SIZE AND FIBRE DENSITY IN REINNERVATED MUSCLE.

B. REINNERVATED

| | TYPE | TETANIC FORCE (mN) | MAXIMUM TERRITORY ($mm^2$) | FIBRE DENSITY (cells/$mm^2$) |
|---|---|---|---|---|
| | SO | 30.1 | 0.45 | 157.30 |
| | SO | 73.8 | 4.14 | 36.92 |
| | FOG | 24.5 | 1.66 | 38.02 |
| DEEP | FOG | 29.0 | 0.43 | 168.98 |
| | FOG | 112.2 | 4.27 | 48.67 |
| | FOG | 126.7 | 2.51 | 85.12 |
| | Fint | 86.0 | 4.34 | 29.50 |
| | mean | 68.9 | 2.54 | 80.64 |
| | SD | 38.9 | 1.62 | 54.86 |
| | SE | 14.7 | 0.60 | 20.73 |
| | SO | 47.4 | 3.05 | 22.94 |
| | SO | 48.5 | 2.97 | 26.91 |
| | FOG | 36.8 | 0.64 | 115.30 |
| | FOG | 51.4 | 4.57 | 21.90 |
| | FOG | 174.0 | 3.78 | 46.84 |
| | Fint | 35.7 | 2.29 | 44.03 |
| SUPERFICIAL | Fint | 75.2 | 3.44 | 34.86 |
| | Fint | 116.9 | 1.14 | 193.69 |
| | Fint | 158.8 | 4.49 | 54.53 |
| | FG | 114.1 | 10.99 | 13.64 |
| | FG | 198.3 | 3.06 | 74.49 |
| | FG | 266.3 | 3.17 | 67.24 |
| | FG* | 278.1 | 9.79 | 38.21 |
| | mean | 123.2 | 4.11 | 58.04 |
| | SD | 82.3 | 2.90 | 47.05 |
| | SE | 22.8 | 0.80 | 13.05 |

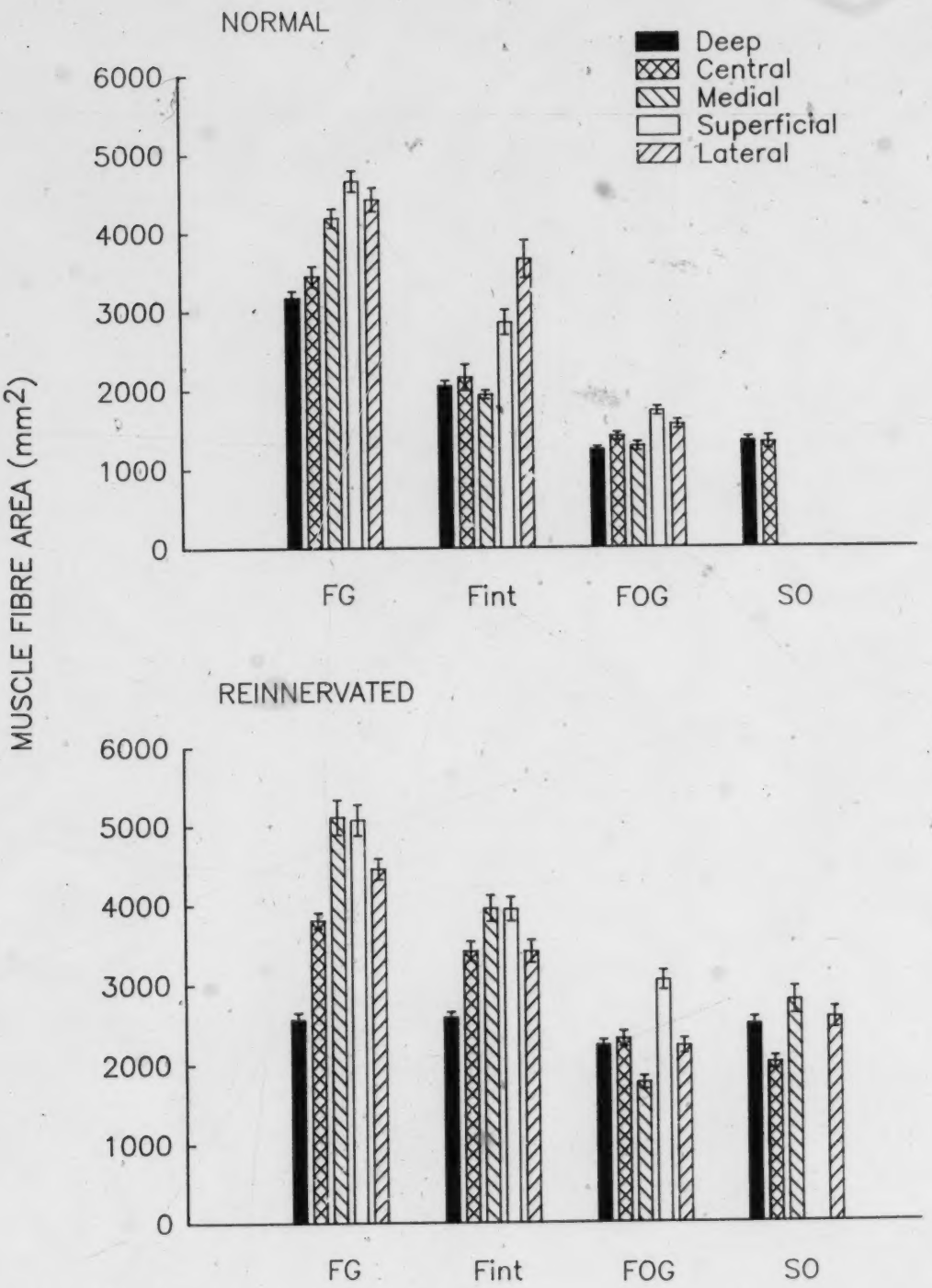
region however the variability in fibre density is so high that no trend is apparent.

### 5.III.5 Muscle Fibre Areas

The results from cross-sectional area measurements confirm the presence of a deep to superficial size gradient in the TA muscle, previously reported by Pullen (1977) and Armstrong and Phelps (1984), and demonstrate its return in reinnervated muscle (Figure 5.5). In normal muscle all fibre types were smallest in the deep region as expected, and the fast types were largest in the lateral and superficial locations. In reinnervated muscle the same trends are apparent. Pullen (1977) has reported a 9-fold range in type SO fibre areas and an 8-fold range in both FOG and FG fibre areas sampled from all regions of the muscle. In the two muscles examined here the range in sizes were considerably smaller in the control animal (3- to 4-fold) except for the Fint type which was 6-fold. In the reinnervated muscle the areas of all types varied over a 5- to 6-fold range.

In reinnervated muscle the fact that the size gradient is maintained suggests that positional factors exert a strong influence on fibre size. This could explain the previous observation (Chapter 2, Figure 2.10) that fibre sizes within reinnervated units vary over a broader range than normal. It appears that a fast motoneurone cannot make small fibres larger and likewise a slow motoneurone cannot make large fibres smaller. This apparently dominant effect of position has

Figure 5.5 Regionalization of Muscle Fibre Size. Muscle fibre cross-sectional areas were measured from two areas in each of five 5 regions: deep, central, medial, superficial, and lateral from a normal (A) and reinnervated (B) muscle. A gradient in size from small to large as one moves from deep to superficial regions is evident in both muscles. The muscle weights of these two animals were 580 mg and 600 mg for normal and reinnervated, respectively and body weights were 292 g and 352 g. These muscles were chosen on the basis of their similarity in weight and on the fact that the tissues were well preserved on all histochemical stains in all areas of the muscle.


NORMAL
Deep
Central
Medial
Superficial
Lateral
6000
5000
4000
3000
2000
1000
0
MUSCLE FIBRE AREA (mm2)
FG
Fint
FOG
SO
REINNERVATED
6000
5000
4000
3000
2000
1000
0
FG
Fint
FOG
SO

the result that fibres tend to recover their former size regardless of the source of innervation.

## 5.IV DISCUSSION

The results from this study support previous reports of compartmentalization within muscles (English and Weeks, 1984; Iliya and Dum, 1984; Balice-Gordon et al., 1988) and indicate that compartmentalization is preserved after reinnervation. The normal regionalization of muscle fibre types with respect to the frequency of occurrence is lost in reinnervated muscle however the gradient in fibre size (which increases from deep to superficial) appears to return.

It was stated earlier that the parent nerve as it enters the muscle divides into two primary branches. To avoid confusion of terminology, the term 'fascicular division' will be used when referring to primary branch points or other branching of the parent nerve that is unaccompanied by branching of individual axons. Nearly all motor units from both normal as well as reinnervated muscle were found to be localized in either the deep or superficial region indicating that individual axons branch distal to the primary fascicular division. The drawings of unit territories in the cat TA from Bodine et al. (1987; see Figure 3) also show little overlap between these regions. Territory size was found to correlate well with force output and since force varies with axonal diameter (Gordon and Stein, 1982; Gordon et al., 1986) it suggests that the extent of the final state of axonal branching (ie, following removal of multiple synapses) is also dependent on size.

### 5.IV.1 Distribution Patterns in Normal Muscle Units

From visual inspection the distribution of fibres in normal muscle units is diffuse and adjancies are observed to be relatively rare. In 14 of the 20 units the density of fibres appeared to be highest in the centre of the territory and decreased towards the territorial boundary, as previously reported by Edstrom and Kugelberg (1968) while the remaining units did not show this trend. Analysis of minimum and maximum territorial areas revealed an irregular distribution of fibres around the outermost limit such that relatively large expanses of the territory were devoid of fibres. In support of these observations, Pfeiffer and Friede (1985) have shown that the density of fibre branching is highly variable from region to region along the length of axons supplying the rat medial gastrocnemius muscle. Thus although the patterns of fasciculation and branching of outgrowing neurites as well as the patterns of growth and ramification of nerves into muscle are highly ordered and reproducible (Landmesser, 1984; Dahm and Landmesser, 1988), major rearrangements must occur during maturation to result in such irregular spacing of both muscle unit fibres and axonal branches.

Based on the present results and those from Bodine et al. (1988) it would appear that the spacing of normal unit fibres is less stereotyped than has previously been described. Interestingly, one unit in Bodine's study which had

fewer adjacencies than random, which is suggestive of active segregation, was type SO. It was noted previously that the two relatively diffuse units observed in reinnervated muscle (see Figure 5.3 B) were both type SO. Of the two SO units in normal muscle, one was widely spaced with a fibre density of about 6 cells/$mm^2$, and the other was more compact with a density of 15 cells/$mm^2$. Kugelberg (1973) has shown that motor unit fibres in the rat soleus muscle, which is predominantly slow, are much more widely spaced than in the TA and can cover up to 75% of the total muscle cross-sectional area. Since the process of synapse removal during the neonatal period is known to increase with activity (O'Brien et al., 1978; Benoit and Changeux, 1978) and slow motor units fire more often than fast ones (Hennig and Lomo, 1985) it follows that relatively more synapses could be eliminated from these immature slow motor units and the final distribution pattern of their muscle fibres could become more diffuse as a result. The above argument may explain the apparent density differences between fast and slow muscle units but it cannot account for the irregular distribution patterns of the muscle fibres. Dahm and Landmesser (1988) have recently shown striking differences in the branching patterns of axons growing in the vicinity of fast and slow myotubes in chick embryos. Since avian slow muscle receives distributed innervation, it would be interesting to determine if the developmental branching pattern is different between axons growing on mammalian fast and slow myotubes.

### 5.IV.2 Reinnervated Muscle Units

The distribution of muscle unit fibres in reinnervated muscle is frequently described as highly clumped or tightly grouped. We have shown that this is not the most common spatial pattern. Rather, the unit fibres most commonly appear in groups of clumps with occasional scattered fibres and within these clumps fibre density is highly variable. Fibre density within the minimum territories ranged from 97 to 285 cells/mm$^2$. It seems likely that these spatial patterns arise from more extensive branching at more proximal levels while the tightly packed units result from local terminal sprouting. The observation that units are located in one or the other of the deep and superficial regions indicates that at least the primary fascicular division pattern is maintained. It was mentioned previously that in normal muscle, axonal branching coincides with the fascicular divisions distal to this point (Pfeiffer and Freide, 1985) and recent work by Landmesser and colleagues (1988) has shown that during development the relative amounts of N-CAM and Ng-CAM determine the patterns of fasciculation. Following axonal injury regenerating axons as well as denervated muscle fibres re-express N-CAM on their surfaces (Covault and Sanes, 1985). During regenerative growth axons follow the degenerating axon pathways left behind apparently by adhering to the Schwann cell basal lamina. Soluble factors emanating from the proliferating Schwann cell population in the distal stump appear to induce the injured axon

to grow towards it (Longo et al., 1983; Keynes, 1987). Furthermore, these axons form synapses preferentially at the original synaptic sites (Gutmann and Saunders, 1944; Gorio et al., 1983). The fact that all reinnervated units are clumped into one or more groups, indicates that regenerating axons do not follow all the branch points of empty intramuscular endoneurial tubes left behind by the previous axons that have degenerated. It is unknown what differences in adhesivity are present in the intramuscular structures to result in some axons branching relatively widely and others branching only locally. Either the integrity of the smaller tubes is not well maintained, or the regenerating axons do not respond appropriately to the local cues they re-express. It is possible that the denervated muscle fibres provide a more compelling stimulus for branching such that axons are drawn outside their presumed fascicular boundaries to reinnervate them. This suggestion is supported by the observation that the concentration of N-CAM is more highly concentrated on interstitial cells near denervated endplates than on regenerating axon terminals or muscle fibres (Sanes and Covault, 1986).

Clearly, further work is needed to understand what factors are determining the initial pattern of branching during reinnervation. The nature of the competitive and non-competitive processes occurring during the removal of multiple synapses are also poorly understood at the motor unit level both during normal maturation and reinnervation. With the presently available biochemical tools adhesive substances known to be re-expressed after denervation can be systematically examined to

determine the timing of their expression as well as the nature of their influence on branching of reinnervating nerves. Since one does not see reinnervated motor units developing force far outside the normal range, it suggests that the number of terminal arbors a motoneurone can support is an intrinsic property which is not lost after injury.

# CHAPTER 6

# GENERAL DISCUSSION

The results from this study have implications for three issues that have been central to motor unit physiology: 1) size dependent branching, 2) plasticity and 3) neural control of muscle properties. Questions concerning the anatomical, mechanical, electrical and biochemical properties of motor units have occupied scientists since the concept of the motor unit as the smallest functional element of motor control was first introduced by Liddell and Sherrington (1925). Since that time it has been established that muscles contain units with a range of size related properties well matched to their functional usage and show a high degree of plasticity allowing adaptive changes to altered functional demands.

Perhaps the most controversial issues have been the functional significance of cell size and the degree of neural control over muscle properties. In this study, evidence has been presented that large motor units contain more and larger muscle fibres which implies that motoneurone branching is size dependent, as originally proposed by Eccles and Sherrington (1930). The direct fibre counts and area measurements from glycogen depleted motor units have shown that differences in the number and size of muscle fibres together account for nearly all of the variation in force between units in normal and reinnervated muscle. Accordingly, the differences in specific tension between units is small, which

agrees with previous direct measurements on single fibres (Lucas et al., 1987) indicating that fast and slow motor units possess the same force generating capacity. When these parameters were estimated indirectly, the same general trend emerged in spite of the fact that IR values were not always numerically similar to those obtained directly. Since motoneurones are most often recruited in an orderly fashion from small to large during voluntary movement, these results further imply a recruitment order of force which is determined by both the extent of branching and the size range of muscle unit fibres.

These results conflict with previous indirect studies where differences in IR between units that differed greatly in force production were quite small. The result of this is that IR and fibre area account for considerably less of the variation in force and specific tension for considerably more. It is suggested that the large number of possible errors involved in estimating these parameters indirectly is the cause of these opposing results. Included in these errors are the effects of averaging force and fibre areas of like type which can obscure differences within types. The fact that the type S motor unit population in the rat TA is small and few are sampled physiologically, and that the SO fibre population is also small so that few are sampled histochemically, may explain the reasonably good agreement between direct and indirect determinations of IR in this study in spite of the potential for significant errors in classification.

After reinnervation, the force range of the motor unit population is similar to normal. This occurs in spite of the fact that the motoneurone population is apparently reduced. In this case size dependent branching is even more striking as the motoneurones branch more extensively to recover muscle force. The number of muscle fibres per motoneurone consistently increases in all unit types by a proportional amount. Thus the mechanisms controlling the final branch number during development seem to also operate during reinnervation.

It is clear that many muscle properties are influenced by the nerves that innervate them and are readily altered by changes in activity patterns (Buller et al., 1960; see also reviews by Pette and Vrbova, 1985; Edstrom and Grimby, 1986). The fact that fibre areas within individual motor units vary over a broad range indicates a limit to this neural control. Yet intraunit fibre area variation is less than that for the whole population of fibres of like type which suggests that the nerve is at least influencing the range of fibre sizes. It is now recognized that muscle fibres within individual motor units are heterogeneous in their properties but the extent of this heterogeneity is controversial. In the present study, it was observed that fibre areas often span a broader range than normal, fatigability is no longer correlated with force, and the twitch-tetanus ratio becomes essentially constant for all unit types after reinnervation. Thus the reinnervating motoneurone has a more limited ability to control the range of size and metabolic properties of its muscle fibres and is unable to respecify muscle properties associated with

activation. This results in a greater degree of heterogeneity within units. However, the myosin ATPase staining intensities in these units are uniform thus the properties associated with contraction speed are converted to match their new nerve supply. This confirms the results from previous histochemical (Kugelberg et al., 1970; Brandstater and Lambert, 1973; Dum et al., 1985) and immunocytochemical (Gauthier et al., 1975) studies on reinnervation. This restoration of contractile properties appropriate to the size of the motoneurone and the observation that these experimental animals had no functional deficit demonstrates a high degree of plasticity.

Since larger force producing motor units contain more muscle fibres and EMG amplitude is thought to be proportional to the number of active fibres, it was expected that EMG should vary predictably with force. Under our recording conditions EMG amplitude was positively correlated with force as expected however the variability in the data was large, particularly for reinnervated units. It was initially thought that this variability could be due to the different spatial locations of units within the muscle such that the deeper lying units were contributing less to the EMG signal than the more superficially located units. This effect could be expected to be greater in reinnervated units where territory size is more restricted and indeed, the variability in EMG was higher in reinnervated muscle. However, spatial location had no effect on EMG in either normal or reinnervated muscle. Fibre density on the other hand seems to be an important

factor since the increased density of reinnervated muscle unit fibres had the effect of enhancing the EMG signal for a given force level. Ultimately, EMG seems to be a rather poor predictor of motor unit size and factors other than the number and size of muscle fibres are more important in determining the magnitude of the recorded electrical activity.

Further examination of muscle unit spatial location revealed that motor units are compartmentalized in the rat TA. In both normal and reinnervated muscle motor unit territories were located either in the deep or superficial regions of the muscle which implies that axonal branching in both cases begins distal to the primary branch point where the nerve enters the muscle. Thus, the processes controlling branching at this level are maintained after injury. At more distal levels however the branching patterns are dramatically different from normal. Reinnervating nerves do not re-establish the normal widespread and scattered branching pattern but seem to give rise to 2 or 3 proximal branches each of which branches more distally to form connections with groups of contiguous muscle fibres. These groups or clumps of fibres may be close together or separated by relatively large distances. It has been suggested that during development differences in spatial and temporal expression of adhesion molecules determine whether an axon will branch away from its neighbours or remain fasciculated and that this results in the highly ordered branching pattern (Dahm and Landmesser, 1988). During reinnervation the adhesion molecules

present during development are re-expressed on the growing terminals as well as on denervated muscle fibres and interstitial cells (Covault and Sanes, 1985). It is possible that their spatial and/or temporal expression is inappropriate to exert the level of control necessary for a more distributed branching. It should be noted that although normal muscle unit fibres are scattered relatively diffusely throughout their territories, the distribution in some units was quite irregular such that there were regions devoid of fibres - occasionally even in the centre of the territory.

In summary, it appears that in anatomical terms, the neuromuscular system is organized such that small motoneurones have lower innervation ratios and the muscle fibres they innervate are smaller, while larger motoneurones have higher innervation ratios and the muscle fibres they innervate are larger. After nerve injury and subsequent reinnervation the same pattern is re-established therefore the factors controlling the expression of size are preserved. Functionally, this range in cell size and the range of terminal field size associated with it results in a range of force capacity of the motor units within a given muscle so that there can be a smooth gradation of force as more units are progressively recruited. Contractile and metabolic properties of motor units are essentially continuously distributed over their respective ranges in a size dependent manner. Cell size therefore, appears to be a fundamental organizational principle in the neuromuscular system.

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